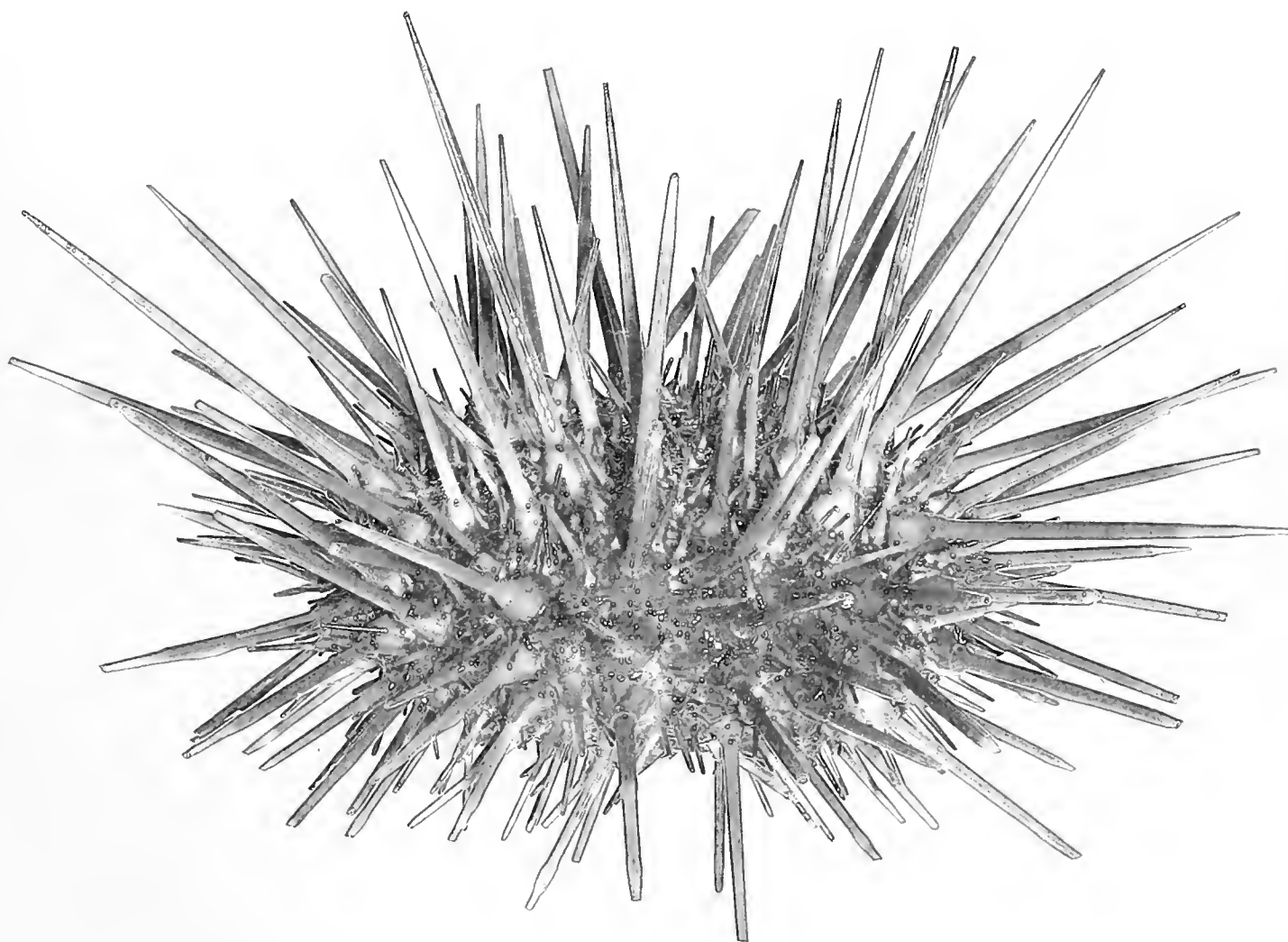


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## THE WHELK DREDGE FISHERY OF DELAWARE

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**ABSTRACT** The status of the Delaware whelk dredge fishery was assessed from state landings reports and from data collected by an onboard observer. Mean annual whelk landings for 1994 to 2000 were 18.5 mt of meats and increased to 241.6 mt for the period 2001 to 2004. Annual landings for 2001 to 2003 were 88% to 119% of the sum of dredge and trawl fishery landings reported by the remaining states on the United States Atlantic coast. Although knobbed whelks, *Busycon carica*, are landed throughout the year, 88% of pooled monthly landings for 1994 to 2004 were taken between March and June inclusive. Eleven observer cruises were conducted March to June 2004. Total estimated daily take of legal (length  $\geq 127$  mm) knobbed whelks ranged from 197–3,710 organisms. Expected catch rates were estimated at 83.2 legal knobbed whelks per hour per meter of dredge width. Dredges were generally towed parallel to bathymetric contours and adjacent to shoal features in 2–20 m depths. Estimates of the area of bottom dredged on each cruise ranged from 4.9–14.2 ha. At times, multiple boats were observed dredging the same bottom, suggesting a cooperative fishing technique. Seventy-nine percent of dredge tows occurred on bottom comprised of mud and sand, and mean numbers of knobbed whelks in 0.04 m<sup>3</sup> samples were greatest from mud/sand habitats. Twenty-one taxonomic groups of organisms were observed in samples taken from dredges. Knobbed whelk and horseshoe crabs, *Limulus polyphemus*, dominated dredge catch by number. Whelks collected in the Atlantic Ocean were significantly larger than those collected in the Delaware Bay. Mean lengths of whelks collected in the Bay in 2004 were significantly less than lengths of Bay whelks collected in 1994 prior to the inception of the minimum length regulation. Shifts in size distribution and the cyclical nature of other whelk fisheries suggest that the elevated landings experienced during 2001 to 2004 are not sustainable.

**KEY WORDS:** knobbed whelk, *Busycon carica*, whelk landings, fishery observer investigation, essential fish habitat, Delaware Bay

### INTRODUCTION

Knobbed whelks, *Busycon carica* (Gmelin, 1791) and channeled whelks, *Busycotypus canaliculatus* (Linnaeus, 1758), are large gastropods that occupy estuarine and shelf habitats from Cape Cod to Cape Canaveral on the Atlantic Coast of the United States (Edwards & Harasewych 1988). Both species occupy sandy habitats and prey on bivalve mollusks (Magalhaes 1948, Carriker 1951, Davis 1981, Peterson 1982). Colloquially known as conchs, the two species have supported multiple fisheries on the United States Atlantic coast at least since the 1930s (MacKenzie 1997). Channeled whelks are harvested primarily with pot or trap gear, whereas knobbed whelks are most vulnerable to dredge or trawl gear (Eversole & Anderson 1984, Davis & Sisson 1988, Walker et al. 2003, Logothetis & Beresoff 2004).

Mark and recapture studies have demonstrated that knobbed whelk growth is generally slow (Anderson et al. 1985, Kraeuter et al. 1989). Short-term negative growth has been observed (Magalhaes 1948, Anderson et al. 1985), and there are examples of periodic rapid growth (DiCosimo & DuPaul 1985, Kraeuter et al. 1989). Average growth in length of 104 marked whelks in eastern Virginia that exhibited positive growth was 3.2 mm/yr (Kraeuter et al. 1989). Knobbed whelk length at age has been recorded from captive specimens and from examination of annual growth marks identified on opercula. Average length ( $n = 20$ ) of 10-y-old captive eastern Virginia whelks was 144 mm (Castagna & Kraeuter 1994). Eastern Virginia whelks 158 mm long were predicted to be 10 y from operculum age data (age =  $-10.99 + 1.33(\text{length})$ ;  $n = 45$ ,  $r^2 = 0.50$ ,  $P = 0.0001$ ; Kraeuter et al. 1989).

Age or size at reproductive maturity in knobbed whelks has been difficult to determine because gender ontogeny has not been well understood. Phenotypic gender differentiation in whelks is determined by the presence or absence of a penis (Magalhaes

1948, Gendron 1992, Castagna & Kraeuter 1994, Power & Keegan 2001, Avise et al. 2004). In addition to having longer shells than males (Magalhaes 1948, Anderson et al. 1985, DiCosimo & DuPaul 1985, Walker 1988), female knobbed whelks are wider and heavier than males (Anderson et al. 1989). Observations of intertidal mating activity indicate that smaller males and large females successfully produce egg strings (Power et al. 2002). Large females, variable and skewed sex ratios (Anderson et al. 1985), and the presence of male characters in females in at least 12 Buccinidae genera (Power & Keegan 2001) has lead to the hypothesis that knobbed whelks may be protandric hermaphrodites that initially mature as males and then undergo functional sex reversal at larger sizes (Walker 1988). Recent DNA analysis, however, indicates that knobbed whelks are genetically dioecious, having mammalian-like sex chromosomes (Avise et al. 2004); observed phenotypic sex reversal or pseudohermaphroditism, may result from a variety of environmental factors (Jenner 1979).

Whelks have been landed as by-catch or from directed dredge or trawl fisheries from all US Atlantic states except Florida (NOAA Fisheries 2004; <http://www.st.nmfs.gov>). Coast-wide landings from dredge and trawl fisheries have increased from 105.2 mt of meats in 1950, when Federal recording began, to a high of 1913.5 mt in 1994. Although total landings have decreased to 549.8 mt in 2003, ex-vessel price has remained high, as total landings value was \$1.55 million in 2003 relative to \$1.79 million in 1994 (NOAA Fisheries 2004). Whelk meats are generally removed from shells locally and then shipped on ice to northern United States metropolitan areas for canning or fresh retail sale; they are also frozen and shipped to East Asia (Eversole & Anderson 1984, Kaplan & Boyer 1992).

In Delaware, an expanding dredge fishery has raised concern over the long-term sustainability of whelk stocks. Additional attention has focused on by-catch considerations and potential physical effects of dredging activity on ecologically significant benthic habitats, particularly the structures created by the colonial poly-

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chaetes *Sabellaria vulgaris* (Verrill, 1873) and *Hydroides dianthus* (Verrill, 1873). Because of the general lack of published information on mid-Atlantic whelk fisheries, this study presents updated landings information and the results of an onboard fishery observer investigation. Temporal and spatial variation in Delaware landings are described and compared with coastwide landings trends to assess the relative magnitude of the fishery. Direct fishery observations document previously unknown attributes such as gear types, spatial and temporal variation in catch and effort, by-catch composition, and variation in the size structure of Delaware knobbed whelk populations. This work focuses on Delaware's dredge fishery, but contextual comparisons are made with dredge, trawl and pot fisheries in other Atlantic States.

## METHODS

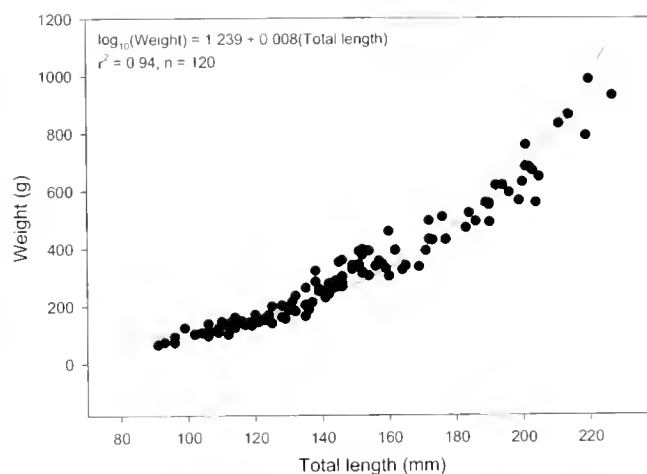
### Landings Analyses

Landings from the Delaware whelk dredge fishery for 1994 to 2004 were summarized from reports by fishery participants. By statute, Delaware commercial dredging license holders are required to submit monthly catch reports that are archived by the Division of Fish and Wildlife. The information recorded includes number of trips, general location dredged, gear type, the monthly take of knobbed and channeled whelks and whether take was by-catch from other fisheries or from directed whelk fisheries. In addition to requiring monthly logbook reporting, Delaware fishery regulations include the possession of a commercial license, a 127.0 mm minimum length limit for knobbed whelks, a 152.4 mm minimum length limit for channeled whelks and a prohibition of night-time dredging.

Whelk landings are published in biannual summaries of state-wide shellfish harvest and fishery status that date back to 1994 (Whitmore & Greco 2005). Current whelk landings information compiled by the state of Delaware were compared with federally reported coast-wide dredge and trawl fishery landings to assess the relative magnitude of the Delaware fishery. Coast-wide landings data provided by NOAA Fisheries (<http://www.st.nmfs.gov>) do not differentiate knobbed and channeled whelks; therefore for comparative purposes, Delaware landings data used here include both taxa. Delaware landings are reported as total weight including the shell, whereas coast wide landings are reported as weight of edible meats; to allow comparison in equivalent units, Delaware landings were divided by a factor of 3 (Whitmore & Greco 2005). Fishery seasonality was assessed from mean monthly landings and catch-per-unit-effort (CPUE) from Delaware harvest reports 1994 to 2004. CPUE was calculated by dividing monthly harvest in weight by the number of trips reported by each fishery participant.

### Onboard Fishery Observation

Eleven observer cruises were conducted on three vessels during March to June 2004 to obtain fishery information not contained in monthly reports. Dredge type, width, ring and mesh size were recorded at the beginning of each cruise. For each dredge tow, start and end times were recorded to determine tow duration and fishing effort. Total daily take, in volume of legal knobbed whelks, was recorded at the end of each cruise. Landed volume was converted to numbers with a conversion factor of 64.37 legal whelks per  $0.04 \text{ m}^3$  (1.0 bushel). Estimated number of legal whelks was converted to weight with cruise-specific length frequency distributions and a length-weight relationship derived during the cruises (Fig.



**Figure 1. Length-weight relationship derived from Delaware Bay knobbed whelks. The dark line represents back-transformed predicted values.**

1). The presence of other vessels fishing in the same location was also noted throughout each cruise.

Cumulative catch was recorded at random intervals throughout the latter seven cruises, and was used to estimate catch rates for legal knobbed whelk. To describe catch relative to time fished, catch volume was divided by the total dredge width, thus standardizing gear differences among cruises. A general catch rate estimate was derived with a linear regression model fitted to cumulative catch data from cruises five through nine (PROC REG, SAS 2002).

GPS coordinates were recorded at the beginning and end of each dredge tow. Waypoints were then plotted with Geographic Information Systems (GIS) to identify spatial distribution of fishing activity. Straight tracklines, interpolated from waypoints, were used to represent the path of the dredges. Dredge tracklines were layered upon bathymetry to identify relationships between fishing activity and seabed topography. GIS tools were also used to estimate the area dredged. Buffer polygons were created around intersecting tracklines with a diameter equal to the dredge width, and the total dredge footprint or virgin area dredged on each cruise was estimated from the area of the polygons. Spatial data from Cruises 2, 6, 9 and 10 were not included in areal estimates because the vessel's course frequently did not follow a straight line between waypoints.

At the end of each tow the dredge was brought aboard and contents were dumped on deck. A  $0.04 \text{ m}^3$  sample of dredge contents was collected before legal-sized whelks were removed. Seabed composition was documented from sample contents and from material attached to dredge gear. All organisms in samples were enumerated to quantify by-catch composition. Knobbed whelk shell lengths were recorded to assess potential spatio-temporal variation in the size structure of the population. Length distributions from Delaware Bay cruises were pooled and compared with lengths from the Atlantic Ocean. Size distribution data collected in the Delaware Bay during 2004 were compared with fishery-dependent data collected in November 1993 and March-April 1994 prior to when the 127.0 mm length limit was imposed. Significant differences in length distributions were identified with the Kolmogorov-Smirnov (KS) test (Proc NPAR1WAY, SAS 2002). Analysis of variance and Tukey tests were used to compare mean

whelk lengths (Proc GLM, SAS 2002); length data were  $\log_e$  transformed to satisfy least squares assumptions.

## RESULTS

### Fishery Landings 1994 to 2004

In addition to the dredge fishery, Delaware has a smaller but significant pot fishery (Table 1). Whelk landings were not recorded by the State until 1994 when legislation was passed regulating all whelk fisheries. Analysis of monthly reports for the period 1994 to 2004 reveal that participation and landings have increased in recent years. The number of license holders that participated in the directed dredge fishery was 11 in 1994, declined to 2 in 1997, and increased to 19 in 2003. Sixteen license holders participated in the 2004 directed fishery (Whitmore & Greco 2005). Landings of pooled knobbed and channel whelks were relatively low for 1994 to 2000 and then increased to high levels in 2001 to 2004 (Fig. 2). The mean annual landing of whelk meats for 1994 to 2000 was 18.5 mt and for 2001 to 2004 the mean annual landing was 241.6 mt (Whitmore & Greco 2005). Except for the blue crab fishery, whelks provide the greatest ex-vessel value of all Delaware commercial fisheries. Dockside ex-vessel value of dredged knobbed and channeled whelks was \$ 574,645 in 2004. Mean annual whelk landings from the Delaware dredge fishery 2001 to 2003 were 96.4% of mean landings for all dredge and trawl whelk landings 2001 to 2003 from the remaining Atlantic states (NOAA Fisheries 2004; Fig. 2). Although Federal coastal fisheries statistics do not differentiate channeled and knobbed whelk landings, State reports indicate that for 1994 to 2004, knobbed whelks comprised between 78.8% and 99.9% of total annual landings by weight in the Delaware dredge fishery. Most fishery effort occurred in the Delaware Bay during 1994 to 2004,

only 3% of 3,031 reported directed trips that landed knobbed whelks occurred in the Atlantic Ocean.

Knobbed whelk landings showed distinct seasonal patterns. Landings from the directed dredge fishery were reported from all months of the 1994 to 2004 period, but the greatest mean total monthly landings occurred from March to June (Fig. 3). For the directed fishery, mean landings of whelk meats for March to June ranged from 12.9–40.2 mt, whereas mean landings for the remaining months ranged from 0.2 mt to 2.7 mt. Mean monthly whelk by-catch landings from the Delaware Bay winter blue crab *Callinectes sapidus* (Rathbun, 1896) dredge fishery ranged from 0.1–2.9 mt. Higher CPUE during March to May suggested seasonally increased vulnerability to dredge gear rather than increased dredging effort (Fig. 3).

### Fishery Observations 2004

**Catch** Between March 15 and June 24, 2004 eleven observer cruises were conducted on three dredge vessels. Two vessels operated from Bowers Beach, DE, and the other from Lewes, DE. Two used single scallop dredges deployed from the stern of deadrise workboats, the other used a motorized bay schooner with paired toothbar crab dredges deployed from the sides. Scallop dredges were 2.62 m and 3.44 m in diameter; bag rings ranged from 63.5 mm to 88.9 mm in diameter; bag mesh ranged from 50.8 mm to 127.0 mm bar measure. Toothbar dredges were 2.3 m wide, rings were 49.0 mm in diameter, and mesh was 127.0 mm bar measure. Fishing effort was directed at whelks on all cruises, but horseshoe crab, *Limulus polyphemus* (Muller, 1785), and blue crab by-catch was retained on Cruise 1 and 3, hard clam *Mercenaria mercenaria* (Linnaeus, 1758) by-catch was retained on Cruise 2.

Whelk dredging began at daylight and ended between 1500 and 1700 h. The general dredge-fishing consisted of towing the gear for approximately 15 min, retrieving the gear, making a 180° turn

TABLE 1.

Coastal Atlantic whelk landings from directed and by-catch fisheries for 2003. Knobbed and channeled whelk landings are pooled. Delaware landings come from monthly logbook reports, whereas landings for the remaining states were reported by NOAA Fisheries.

State	Landings (tonnes meats)													Minimum Legal Size (mm) <sup>4</sup>
	Total Landings	Conch Dredge	Crab Dredge	Clam Dredge	Other Dredge <sup>1</sup>	Crab Trawl	Fish Trawl	Other Trawl <sup>2</sup>	Conch Pot	Blue Crab Pot	Lobster Pot	Fish Pot	Other Pot <sup>3</sup>	
Connecticut	12.6						1.0		10.8		0.7	<0.1		None
Delaware	353.9	157.0	105.5						91.4					127/152 <sup>5</sup>
Georgia	40.9					39.7				1.2				None
Maryland	91.2			<0.1			10.6	<0.1	80.0		0.4	0.20		152/152
Massachusetts	110.0	0.2		0.1	<0.1		17.4		0.6		0.8	<0.1	90.9	70/70 <sup>6</sup>
New Jersey	196.7		163.0	1.3	0.3		2.1		24.6	5.4		<0.1		127/127
New York	30.7						3.1		25.9		1.6	0.1	<0.1	None
North Carolina	3.5					0.1	0.1	2.4		0.9				None
Rhode Island	2.9						0.7		1.6		0.1	0.5		None
South Carolina	1.4					0.3				1.1				102/102
Virginia	287.5	31.7	9.0	0.1			15.6	<0.1	224.5	6.6			<0.1	None/140
Total	1131.5	188.8	277.5	1.5	0.3	40.1	50.6	2.5	459.4	15.2	3.7	0.8	91.0	

<sup>1</sup> Sea scallop and undefined dredge fisheries

<sup>2</sup> Sea scallop and undefined trawl fisheries

<sup>3</sup> Eel, undefined crab, and undefined pot fisheries

<sup>4</sup> Length unless otherwise noted

<sup>5</sup> Knobbed whelk/channeled whelk

<sup>6</sup> Width

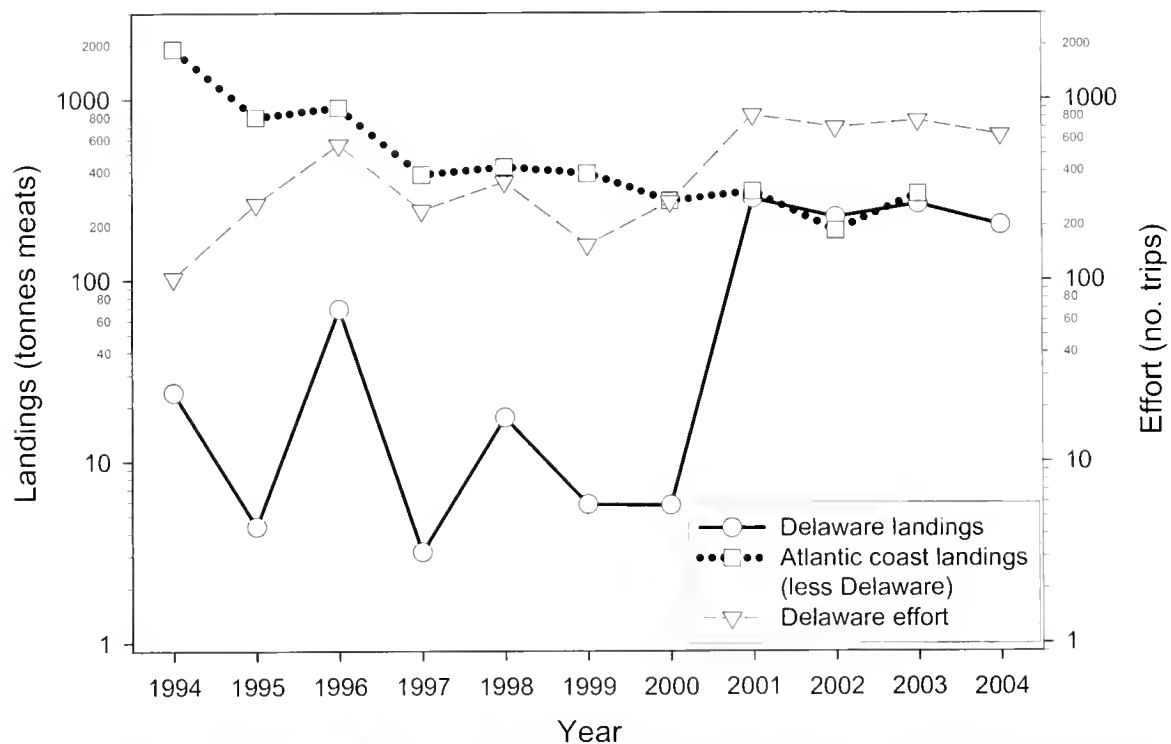


Figure 2. Reported Delaware whelk dredge fishery landings and fishing effort 1994–2004. Delaware landings are compared with the sum of whelk landings from dredge and trawl fisheries in the remaining Atlantic states 1994–2003 as reported by NOAA Fisheries. Knobbed and channeled whelk landings are pooled, as are landings from directed and by-catch fisheries reporting the use of dredge or trawl gear. Note logarithmic scale on vertical axes.

and releasing the gear again. Dredge tows were oriented in the same direction and intersected. Vessel speed during dredging was between 3.5 and 5.2 kts. Except for Cruises 10 and 11, the total number of dredge tows per cruise ranged between 16 and 38

(Table 2). Whelk catch and effort varied among cooperators. Maximum take of legal knobbed whelk occurred on Cruise 4 and was estimated at 2.03 m<sup>3</sup>, 1304.1 kg, and 3677 organisms (Table 2). Higher whelk landings were associated with additional vessels

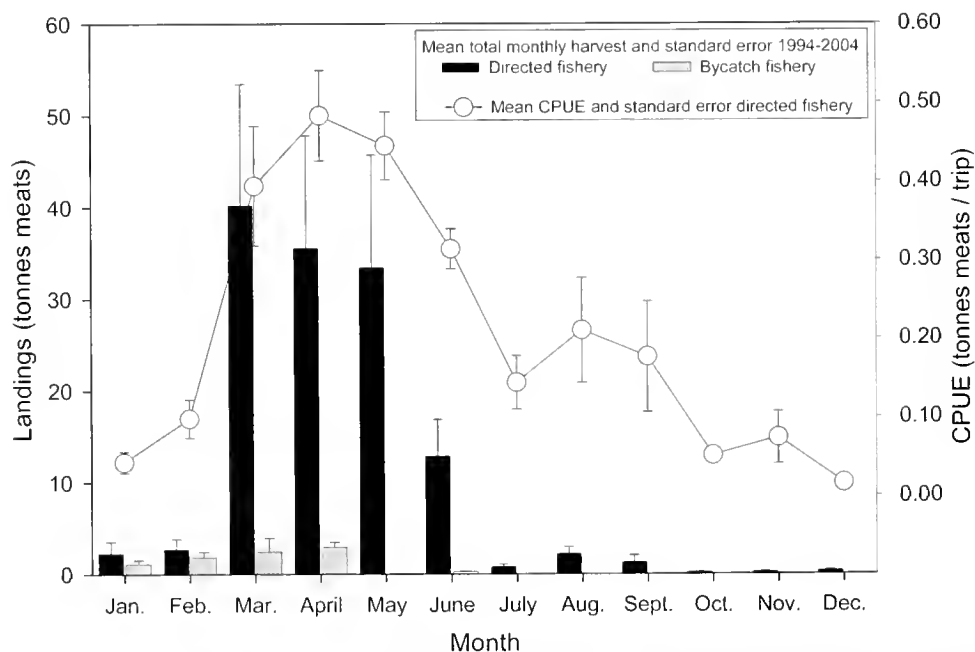


Figure 3. Landings and catch-per-unit-effort (CPUE) seasonality of the Delaware Bay knobbed whelk dredge fishery for 1994–2004. In addition to landings from a directed fishery, whelks are landed as by-catch from the winter blue crab dredge fishery. Note that scales on vertical axes are different.

TABLE 2.

Dredge gear, effort, and landings of legal knobbed whelk ( $\geq 127$  mm) on 11 observer cruises in the Delaware Bay March to June 2004. Horseshoe crab and blue crab were retained on Cruises 1 and 3, and hard clams were retained on Cruise 2.

Cruise <sup>1</sup>	Date (m/d)	Dredge Type	Total Tows	Tow Time	Dredged Area (ha)	Whelk Landed				CPUE <sup>2</sup>	No. Vessels <sup>3</sup>	% Knobbed Whelk in Volumetric Grab Samples (0.04 m <sup>3</sup> )	
						Volume (m <sup>3</sup> )	No.	kg				n	Coefficient of Variation
1 <sup>A</sup>	3/15	Scallop	36	10.8	11.9	1.30	2364	681.4	0.046	0	17	71.6	0.65
2 <sup>B</sup>	3/25	Toothed	31	9.1	—	0.60	1391	446.2	0.014	0	20	7.8	60.24
3 <sup>C</sup>	4/2	Scallop	20	7.5	9.2	0.58	652	199.2	0.023	0	13	58.3	0.84
4 <sup>A</sup>	4/14	Scallop	33	9.3	14.2	2.03	3677	1304.1	0.083	3–12	8	74.5	0.24
5 <sup>C</sup>	4/20	Scallop	17	7.4	12.5	0.98	1773	869.5	0.038	0	14	47.7	0.68
6 <sup>B</sup>	4/30	Toothed	37	9.0	—	1.60	3710	1157.8	0.039	0–6	12	66.0	0.73
7 <sup>A</sup>	5/7	Scallop	38	10.3	9.9	1.52	2758	869.9	0.057	0–4	17	53.5	0.85
8 <sup>C</sup>	5/19	Scallop	16	7.3	12.9	0.79	1445	853.3	0.032	0	14	42.9	0.75
9 <sup>B</sup>	5/27	Toothed	25	8.1	—	1.40	3246	1110.4	0.038	1–3	19	88.1	0.11
10 <sup>B</sup>	6/9	Toothed	11	3.7	—	0.20	464	163.4	0.012	0	6	74.0	0.23
11 <sup>C</sup>	6/24	Scallop	10	3.1	4.9	0.10	197	119.5	0.009	0	9	23.6	3.13

<sup>1</sup> Common subscript indicates same vessel.

<sup>2</sup> CPUE = volume (m<sup>3</sup>) landed/m dredge width/hour dredged.

<sup>3</sup> Number of additional vessels dredging same area

dredging the same area. Catch-per-unit-effort (CPUE) for scallop dredges was 0.009–0.083 m<sup>3</sup>/m dredge width/hour, mean = 0.04; and for toothbar dredges CPUE was 0.012–0.039 m<sup>3</sup>/m dredge width/hour, mean = 0.026 (Table 2). The expected hourly catch

rate derived from Cruises 5–9 (Fig. 4) was 0.05 m<sup>3</sup> legal whelks/m dredge width. The model used to estimate catch rate was (m<sup>3</sup> catch/ m dredge width) =  $-0.03 + 0.05 \times \text{hours}$ ;  $P < 0.0001$ ;  $R^2 = 0.90$ ;  $n = 49$ .

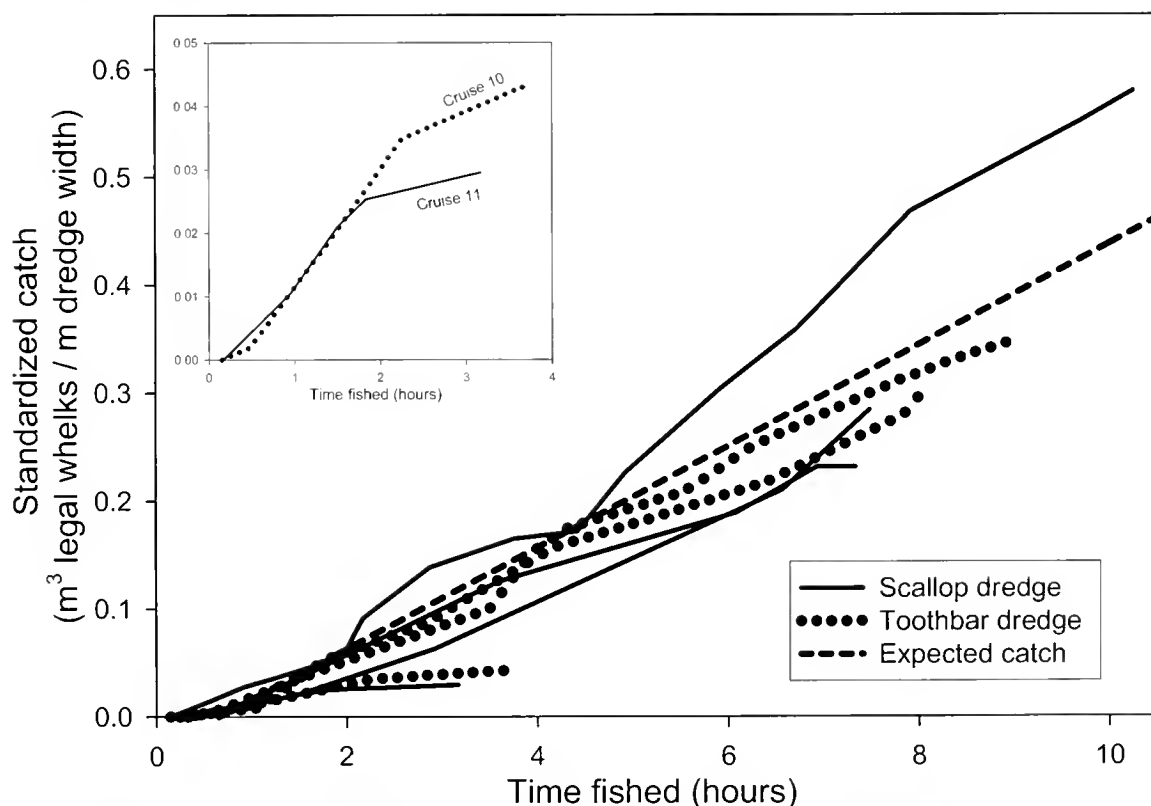


Figure 4. Cumulative catch of knobbed whelks relative to time towed by scallop and toothbar dredges. Catch in volume is standardized by dredge width to allow comparison among cruises and gears. Data presented here come from cruises 5–11. The expected catch rate derived from Cruises 5–9 was 0.05 m<sup>3</sup> of legal whelks per m of dredge width per hour ( $\text{m}^3/\text{m} = -0.035 + 0.047(\text{hours})$ ,  $P < 0.0001$ ,  $R^2 = 0.90$ ,  $n = 49$ ). The inset panel illustrates the late-season decline in catch rates and the cessation of dredging on cruises 10 and 11.

Cumulative catch from Cruises 10 and 11 illustrates why the fishery ceases in late June. On Cruises that occurred prior to 9 June, catch rates remained similar throughout the day (Fig. 4). On Cruises 10 and 11, initial catch rates were similar to previous cruises, but declined after approximately two hours of fishing effort (Fig. 4, inset). Low catch rates and declined efficiency, presumably elicited by increased light levels, prompted both cooperators to cease fishing for the season. June 9 was the last day of dredging for vessel B and June 24 was the last day for vessel C (Table 2); no other boats were observed fishing on either dates.

#### Fishery Location

Dredge-boat cruises occurred in the Delaware Bay and in the Atlantic Ocean (Fig. 5). Cruises 1–7, 9 and 10 were located in the

Bay between Bowers Beach and Cape Henlopen, DE, and Cruises 8 and 11 were located just off-shore, in the Atlantic Ocean, south of Cape Henlopen. Fishery activity occurred adjacent to the beach in the ocean and on the slopes of the numerous shoals that define Delaware Bay bottom topography. Dredge tracks were generally oriented parallel to bathymetric contours (Fig. 6). Depths recorded during dredge deployment and retrieval ranged from 2 to 20 m. The area of virgin bottom impacted by scallop dredges, estimated from dredge trackline distance and dredge width, ranged from 4.9 ha on Cruise 11–14.2 ha on Cruise 4 (Table 2).

#### Benthic Habitat Types

Bottom type was recorded from 212 of 274 total dredge tows. Bottom material observed in dredges included mud, clay, sand,

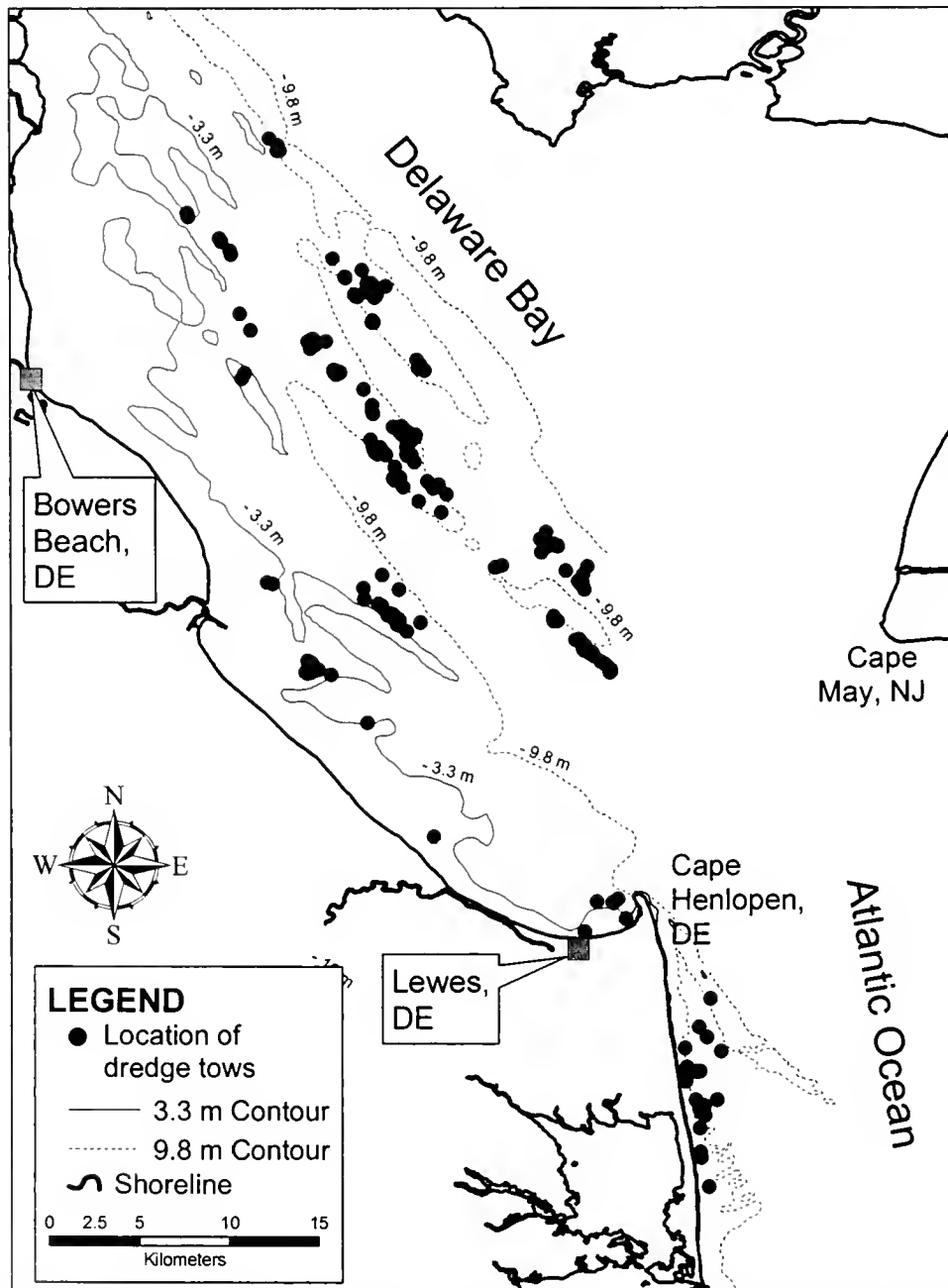


Figure 5. Whelk dredge tow locations recorded from on-board GPS receivers. The parallel Northwest-Southeast orientation of waypoints reflects the orientation of shoal features in the Delaware Bay.

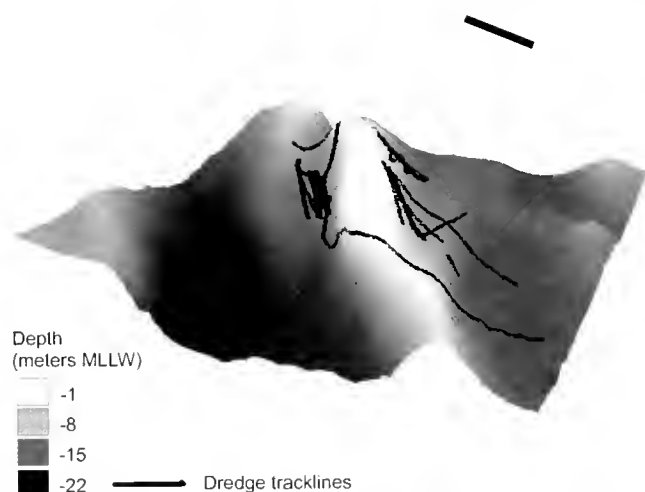


Figure 6. Locations of dredge tracklines relative to bathymetric features at Lower Middle Shoal in the Delaware Bay. GPS coordinates were recorded at the beginning and end of dredge tows. Tracklines are interpolated and assume a straight line between waypoints.

oyster shell, and rock. Less dense material, such as mud, silt and shell fragments may have been washed away during dredge retrieval, and dredges were occasionally rinsed to remove fine sediments, which resulted in potentially ambiguous distinction between Mud/Sand and Sand categories (Fig. 7). Six of the seven bottom categories were characterized by mud or sand components. Knobbed whelks were most frequently associated with mud and sand bottoms. The greatest number of whelks enumerated from  $0.04 \text{ m}^3$  samples came from bottoms classed as mud/sand/shell hash (mean = 24.1; standard error = 3.7,  $n = 8$ ); the lowest number of whelks in samples, came from rock/worm tube bottoms

(mean = 1;  $n = 1$ ; Fig. 7). The most frequently recorded bottom category was mud/sand, at 79% of classified tows, and the least frequent was oyster shell/worm tubes at 0.9% of classified tows. Tube casings of the colonial "reef" building polychaetes *S. vulgaris* and *H. dianthus* were observed infrequently, but were generally associated with hard substrates such as rock and oyster shell.

#### Catch Composition

Proportions of knobbed whelks relative to numbers of by-catch organisms observed in volumetric samples were highly variable. Mean percent knobbed whelk by number was less than 50% on four of the eleven cruises, but ranged up to 88.1% on Cruise 9 (Table 2). Twenty-one taxonomic groups were enumerated from 149 samples taken from dredge contents. The most abundant species in the samples were knobbed whelk and horseshoe crabs (Table 3). Proportions of pooled knobbed whelk by number examined ranged from 31.7% on Cruise 11–88.4% on Cruise 9; for horseshoe crabs proportions range from 1.0% on Cruise 9–41.0% on Cruise 8. The highest proportions of horseshoe crabs by number were observed in samples from ocean Cruises 8 and 11. Knobbed whelks were observed in 98.6% of all dredge tows and horseshoe crabs were observed in 85.2% of all tows (Table 3). Windowpane flounder *Scophthalmus aquosus* (Mitchill, 1815) and unidentified skates were the most evenly distributed taxonomic groups as they were observed in all cruises in similar proportions. Channeled whelk occurred in only 22.2% of the dredge tows sampled and comprised only 1% of organisms observed in samples (Table 3). Proportions of channeled whelk in pooled volumetric samples ranged from 2.9% in Cruise 8% to 0% on Cruise 11; both cruises occurred in the Atlantic Ocean. Although not enumerated in samples, the shells of dead knobbed whelks generally were the most abundant nonliving material observed in dredges, and frequently were more abundant than living whelks.

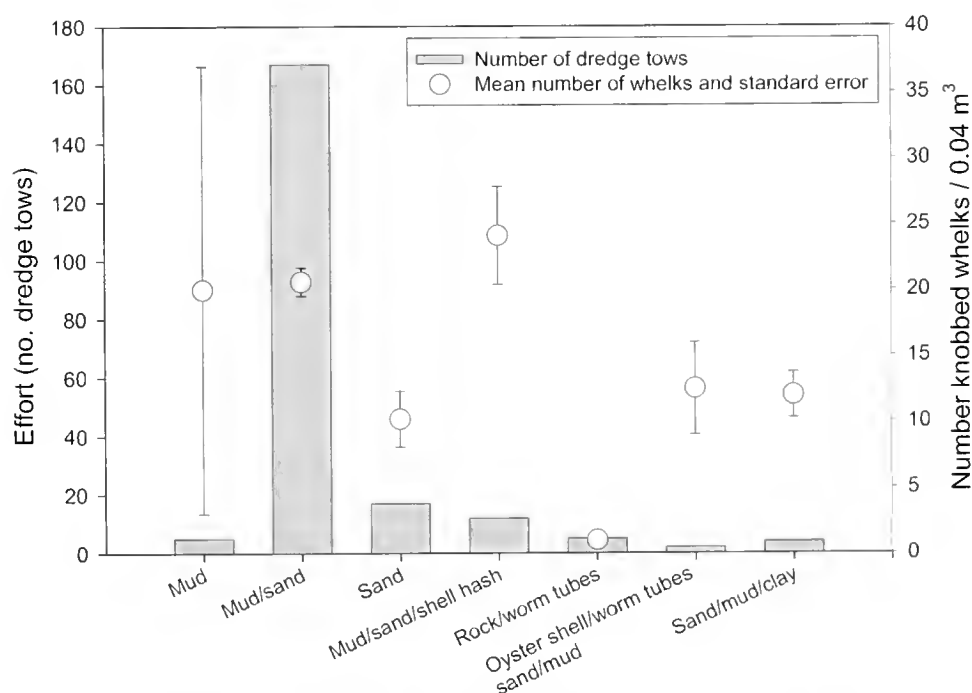


Figure 7. Fishing effort and numbers of sampled whelks relative to benthic habitat types determined from material on the dredge and associated with dredge contents. Knobbed whelks were enumerated from  $0.04 \text{ m}^3$  samples taken from 147 tows. Worm tubes are casings of *Sabellaria vulgaris* and *Hydroides dianthus* polychaetes.

TABLE 3.

Composition of organisms observed in 0.04 m<sup>3</sup> samples taken from whelk dredge contents. Counts were pooled from single samples from 149 dredge tows on 11 vessel cruises. Frequency of occurrence is the proportion of samples containing the specific taxon.

Common Name	Latin Name	Number Observed	Percent of Total	Frequency of Occurrence (%)
Knobbed whelk	<i>Busycos carica</i>	2813	63.96	98.66
Horseshoe crab	<i>Limulus polyphemus</i>	802	18.24	85.23
Skate	Rajidae	181	4.11	51.68
Windowpane flounder	<i>Scophthalmus aquosus</i>	120	2.73	43.62
Hard clam	<i>Mercenaria mercenaria</i>	105	2.39	12.08
Spider crab	<i>Libinia</i> spp.	102	2.32	22.82
Rock crab	<i>Cancer irroratus</i>	87	1.98	23.49
Blue crab	<i>Callinectes sapidus</i>	58	1.32	24.16
Channeled whelk	<i>Busycotypus canaliculatus</i>	44	1.00	22.15
Summer flounder	<i>Paralichthys dentatus</i>	37	0.84	14.77
Blue mussel	<i>Mytilus edulis</i>	15	0.34	2.68
Starfish	Asteriidae	9	0.20	4.69
Mud crab	<i>Panopeus</i> spp.	8	0.18	4.03
Oyster toadfish	<i>Opsanus tau</i>	7	0.16	4.03
Hogchoker	<i>Trinectes maculatus</i>	3	0.17	2.01
Striped searobin	<i>Prionotus evolans</i>	2	0.05	1.34
Atlantic moonsail	<i>Polinices duplicatus</i>	2	0.05	1.34
Dogfish	Elasmobranchiomorphi	1	0.02	0.67
Northern pufferfish	<i>Sphoeroides maculatus</i>	1	0.02	0.67
Squid	Teuthoidea	1	0.02	0.67
Total observed =		4398		

### Size Distributions of Knobbed Whelk

Shell lengths of knobbed whelk taken from volumetric samples ranged from 66–243 mm (Table 4). Mean lengths ranged from 132.5 mm on Cruise 1–185.9 mm on Cruise 11. Although there were significant differences in log<sub>e</sub> transformed mean length among the cruises ( $P < 0.0001$ ), considering spatial and gear differences among cruises, comparison of means indicate whelks had similar lengths on 7 of the 11 cruises (Table 4). Transformed mean length on Cruise 1 was significantly less than all the other cruises ( $\alpha = 0.05$ ; Tukey's Studentized Range Test). Mean length on Cruise 11 was significantly greater than all the other cruises except Cruise 8, and Cruise 8 mean length was not significantly different from Cruise 5 means ( $\alpha = 0.05$ ; Tukey Studentized Range Test; Table 4). Mean lengths from Cruises 2–4, 6–7 and 9–10 were not significantly different ( $\alpha = 0.05$ ; Tukey's Studentized Range Test). The observed variation in whelk size among cruises may be explained by location, as the two cruises with the largest mean whelk length, Cruises 11 and 8 (Table 4), occurred in the ocean.

Because of length differences observed among individual Bay and Ocean cruises, whelk length data from the Bay were pooled and compared with Ocean length distributions. The 2004 length distribution of knobbed whelks collected in the Bay was generally mound shaped (mean = 148.2, median = 147, standard error = 0.54,  $n = 2198$ ), whereas the length distribution from the Ocean

TABLE 4.

Knobbed whelk length statistics recorded from dredge fishery observer cruises conducted in the Delaware Bay and in the Atlantic Ocean 2004.

Cruise	Location	Knobbed Whelk Length (mm)					Similar Means <sup>1</sup>	n
		Mean	Median	Standard Deviation	Min.	Max.		
1	Bay	132.5	128	22.3	87	192	A	298
2	Bay	145.9	151	22.2	88	187	B	185
3	Bay	145.7	142	22.5	93	211	B	211
4	Bay	146.4	145	28.5	84	221	B	185
5	Bay	173.2	174	27.8	113	223	C	193
6	Bay	149.5	149	19.9	96	204	B	267
7	Bay	147.7	144	22.2	98	209	B	360
8	Ocean	176.7	181	33.8	66	243	CD	175
9	Bay	150.0	149	24.1	91	223	B	394
10	Bay	151.6	153	24.9	91	211	B	105
11	Ocean	185.9	193	25.9	107	222	D	72

<sup>1</sup> Equivalent letters indicate that means of log<sub>e</sub> transformed lengths were not significantly different (Tukey test,  $\alpha = 0.05$ ).

was multimodal and skewed towards greater proportions of larger whelks (mean = 179.4, median = 183, standard error = 2.03,  $n = 247$ ; Fig. 8). Bay and ocean length distributions were significantly different (KS test,  $P < 0.0001$ ). Log<sub>e</sub> transformed mean lengths of whelks collected in the Ocean in 2004 were significantly greater than Bay whelks collected in 2004 ( $P < 0.0001$ ).

The relative frequency distribution derived from fishery dependent length data collected from the Bay dredge fishery in November 1993 and May and April 1994 (Michael Greco, Delaware Department of Fish and Wildlife, Little Creek, DE 19961; unpublished data), prior to the inception of the 127.0 mm minimum length limit, was compared with the 2004 Bay distribution (Fig. 8). The two distributions were significantly different (KS test,  $P < 0.0001$ ). Delaware Bay knobbed whelk lengths measured in 1993–1994 (mean = 158.8 mm, median = 147, standard error = 0.64,

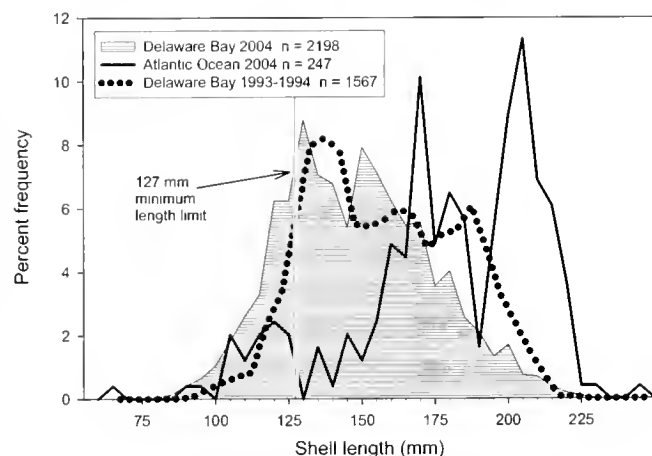


Figure 8. Spatial and temporal comparisons of knobbed whelk length distributions from the Delaware dredge fishery. Length data from the Delaware Bay came from 2004 observer cruises 1–7 and 9–10 and from fishery dependent samples collected in 1993 and 1994 prior to the inception of the 127 mm minimum length limit. Atlantic Ocean lengths were recorded on 2004 observer cruises 8 and 11.



$n = 1567$ ) was intermediate between 2004 Bay lengths and 2004 Ocean lengths. Log<sub>e</sub> transformed lengths were significantly greater than 2004 Bay mean length, but less than 2004 Ocean mean length (Tukey's Studentized Range Test,  $\alpha < 0.05$ ). Length variation suggests a temporal shift toward smaller whelks over the last decade (Fig. 8). The proportion of Bay whelks  $>175$  mm declined from 29.4% of the sample in 1993 to 1994 to 17.2% of the sample in 2004.

## DISCUSSION

### Whelk Fishery Landings

In addition to dredge gear, whelks are landed with trawls, pots, and traps (Table 1). In 2003, pooled landings of knobbed and channeled whelks from all Atlantic dredge/trawl fisheries were 561.3 mt meats, and landings from trap/pot fisheries were 570.1 mt meats (NOAA Fisheries 2004; <http://www.st.nmfs.gov>). The greatest landings from trap/pot fisheries were 224.5 and 91.4 mt meats from the states of Virginia and Delaware respectively; and the greatest landings from dredge fisheries were 262.5 and 163.0 mt meats from the states of Delaware and New Jersey respectively (Table 1).

Over the last decade, landings and effort from the Delaware whelk dredge fishery have exhibited a general increasing trend, but during this period landings from dredge and trawl fisheries the other Atlantic states have declined. Aside from the states of Delaware and New Jersey, the top producing states, Georgia, Maryland, Massachusetts, South Carolina, and Virginia (NOAA Fisheries 2004), are currently experiencing periods of reduced landings (Fig. 9). Whelk landings time series, from 1958 to 2003, indicate that dredge and trawl fisheries generally exhibit abbreviated periods of increased landings followed by longer periods of reduced landings, and that the intervals of reduced harvest may last 1–2 decades (Fig. 9). Because coast-wide landings of knobbed and channeled whelks are pooled, and directed and by-catch fisheries are not differentiated, it is difficult to identify the factors that influence landings cycles. For example, peak landings in Virginia during 1974 to 1975 (Fig. 9) resulted from large landings of channeled whelk by-catch from the surf clam dredge fishery, that coincided with peak surf clam landings (DiCosimo & DuPaul 1985).

Landings variability in whelk dredge and trawl fisheries is determined by a combination of supply, demand, economic costs, or regulatory constraints. In New Jersey, whelks can be harvested as by-catch from the blue crab dredge fishery, and as with any other type of fishery, whelk landings are largely determined by abundance and market conditions (Jeffrey Normant, New Jersey Bureau of Marine Fisheries, Port Republic, NJ 08241; pers. comm.). In Georgia, landings are dependent on whelk abundance, operational costs, and success of the previous shrimp season. The Georgia whelk trawl fishery offers off-season income to the shrimp fleet, and annual shrimp and whelk landings are often inversely related (James Page, Georgia Marine Resources Division, Brunswick, GA 31520; pers. comm.). Similar to the Georgia fishery, landings in South Carolina provide supplemental income to the shrimp fleet during the off-season and fishing effort is dependent on a combination of market value, cost of fuel, and whelk abundance (William Anderson, South Carolina Marine Resources Division, Charleston, SC 29422; pers. comm.).

Among the top whelk producing states in the mid and south Atlantic (Fig. 9), Delaware's is the only directed dredge or trawl fishery that has no seasonal restrictions. Maryland has no whelk

season closure, but trawl landings are by-catch from a groundfish trawl fishery (Steven Doctor, Maryland Department of Natural Resources, Matapeake, MD 21666; pers. comm.). In New Jersey, whelks can be landed throughout the year with pot gear, and can be landed as by-catch from the winter blue crab dredge fishery that occurs from mid-November through mid-April (<http://www.state.nj.us/dep/fgw/saltwater>). In Virginia knobbed whelks are landed as by-catch from the blue crab dredge fishery that occurs between December and March in the Chesapeake Bay. Virginia also allows a directed fishery in distinct inshore and off-shore management zones with varying seasonal restrictions (<http://www.mrc.state.va.us/commercialcrabbingrules>). The South Carolina directed trawl fishery occurs between the shrimp fishery closure in January and the reopening in April, and landings are usually highest in the months of March to April. South Carolina whelk seasons may close prematurely if fishery-dependent information identifies low whelk abundance or presence of sea turtles (William Anderson; pers. comm.). The Georgia trawl fishery also occurs during a January to April shrimp closure; premature closure of the whelk season may be influenced by market forces or triggered by the onset of knobbed whelk reproduction identified by the occurrence of egg strings in fishery independent surveys (James Page; pers. comm.).

There is a strong seasonal pattern in landings from the Delaware whelk dredge fishery. Whelks are retained as by-catch from the blue crab dredge fishery that runs from mid-December through March, and are landed by a directed fishery that is open year-round. The highest mean monthly landings, however, occur March to June. High CPUE demonstrates that seasonality in landings may be related to increased whelk vulnerability to dredge gear during March to June. Behavioral information is largely derived from observations of intertidal whelks, but generalizations may be drawn to explain seasonality in CPUE of the subtidal fishery. Intertidal whelks in Georgia were most active during spring and fall (Walker et al. 2004). Released whelks were buried and inactive at 9°C water temperature (January) and were on the surface or partially buried at 13°C water temperature (March); whelks released in the summer initially burrowed up to 14.4 cm into the substrate and then migrated to subtidal areas (Walker et al. 2004). In North Carolina intertidal whelks released in the summer immediately burrowed into sediments and remained so for several days; burrowing behavior was so prevalent that it was termed a vertical migration (Magalhaes 1948).

Whelk activity appears negatively associated with ambient light intensity; during the summer, intertidal whelks were most active at night, during crepuscular periods, or on overcast days (Magalhaes 1948). During December to February subtidal whelks may be sufficiently buried to be less vulnerable to dredge gear (Fig. 3). Although whelks are active in the summer, negative phototaxis may explain intermediate summer CPUE levels. Commercial dredgers in New Jersey report that both knobbed and channeled whelk burrow during the day and capture success is greatest at night or on days with heavy cloud cover (Dobarro 1993). Negative phototaxis during the summer may explain why catch rates on Cruises 10 and 11 declined after approximately 2 hours of dredging (inset Fig. 4), and resulted in a cessation of fishing activity for the season. High CPUE during March–May may result from sediment emergence and increased diurnal activity mediated by increased temperature relative to winter months, and presumably lower light intensity and shorter photoperiod relative to summer months. Although nothing is published on the seasonality in the

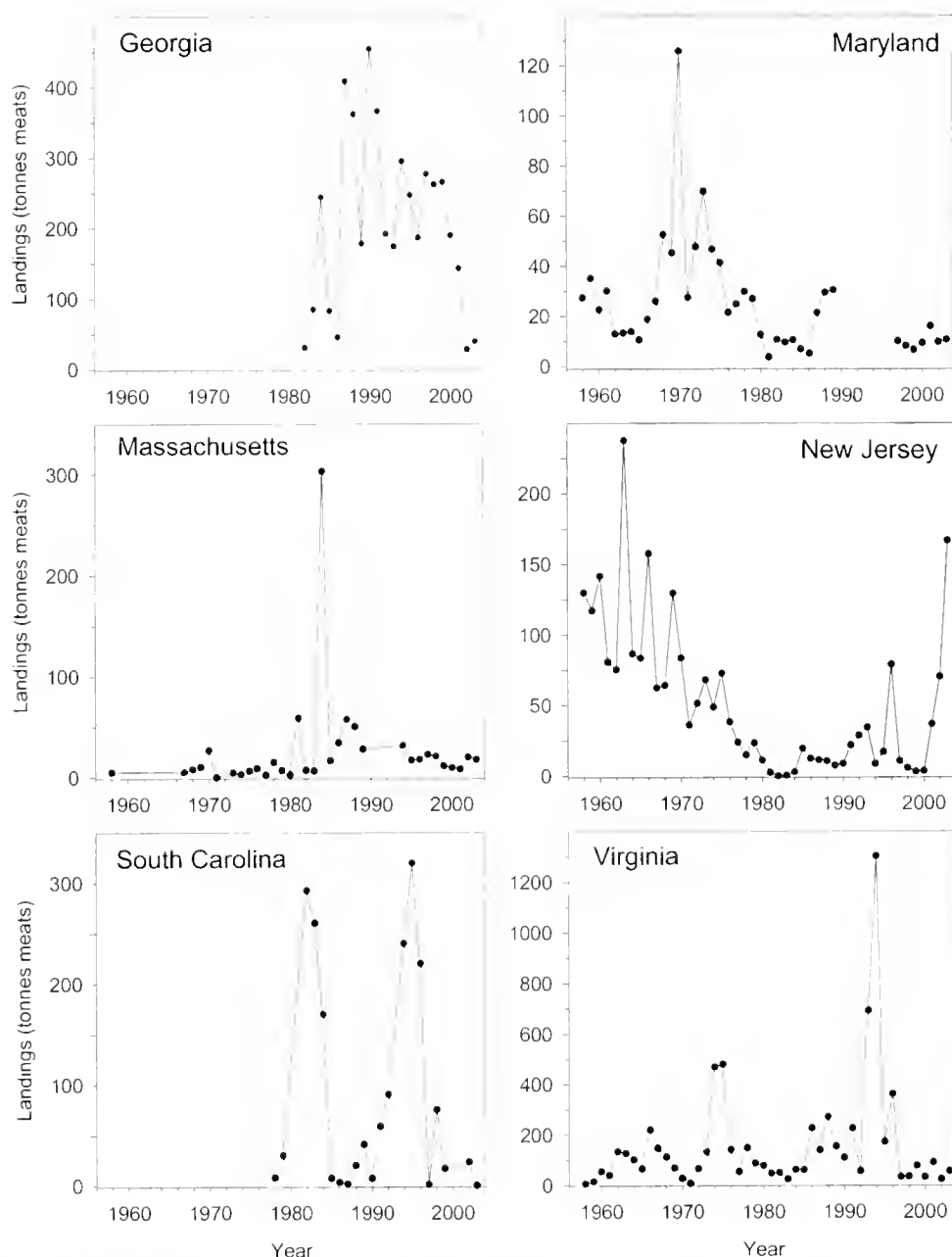


Figure 9. Whelk landings time series reported to NOAA Fisheries by six of the Atlantic states with the highest producing dredge and trawl fisheries. Landings of knobbed whelks and channeled whelks are pooled. Note that vertical axes are not equivalent.

spatial distribution of subtidal whelks in Delaware, high spring-time CPUE may also be related to high densities resulting from affinities to specific over-winter habitats or from premating aggregations.

#### *Gear and Benthic Habitats*

Whelk burrowing behavior may explain fishery tactics and the use of specific gear types. Toothbar dredges are the most frequently used gear in Delaware, and are the traditional gear of the winter blue crab fishery. Currently, only three fishermen use scallop dredges, but use of this gear is likely to increase in the future. Unlike toothbar dredges that have 15 cm teeth, scallop dredges use heavy tickle chains to disturb the substrate ahead of the bag. Although catch data was collected from only one toothbar dredger,

CPUE (Table 2) and cumulative catch plots (Fig. 4) suggest that scallop dredges are at least as efficient as toothed dredges for harvesting knobbed whelks. Gears used in other whelk dredge/trawl fisheries include modified shrimp trawls (Anderson et al. 1985, Walker 1988), groundfish trawls and assorted dredges used to collect clams, sea urchins, mussels and oysters (NOAA Fisheries 2003). Dredgers on the New Jersey side of the Delaware Bay are permitted to use toothed crab dredges, dredges with a toothless bar, or a chain in place of the toothed bar (Paul Scarlett, New Jersey Bureau of Marine Fisheries, Port Republic, NJ 08241; pers. comm.). The spatial pattern of dredge deployment in the Delaware fishery is typified by multiple tows over the same area by single or multiple boats. Presumably, dredge track overlap and cooperative dredging enhance catch rates because whelks are at least partially

embedded in the sediment and are not easily dislodged on the first pass. The use of toothbar dredges and scallop dredges with heavy tickle chains also indicate that whelks must be dug from the substrate. In South Carolina, otter trawls are modified with cables and tickler chains that dig whelks from the sediment, and chaffing gear is added to minimize damage to codend webbing (William Anderson; pers. comm.). Because otter trawls must penetrate into the sediment, gear and boat damage is a major economic consideration for entry into the Georgia fishery (James Page; pers. comm.).

Considerable focus has been given to the effect of mobile fishing gear on benthic habitats (National Research Council 2002). Essential Fish Habitat (EFH) considerations relative to ecologically significant habitats were included in recent fisheries legislation (Benaka 1999). So-called "worm reefs" (structure created by the tube dwelling polychaetes *S. vulgaris* and *H. dianthus*), occur in the Delaware Bay (Wells 1970, Haines & Maurer 1980), and have been attributed to having EFH value (NREFHSC 2002, Miller 2001). Increased effort in the whelk dredge fishery over the last decade has raised concern over potential negative effects on Delaware Bay benthic habitats. *H. dianthus* creates a calcareous casing (Haines 1978) and was only observed from dredge tows that contained rock and oyster shell. *S. vulgaris* creates casings of sand grains (Wells 1970) and when observed in dredges, was attached to a variety of hard materials including the shells of living whelks and horseshoe crabs. Material considered to be representative of worm-reef habitat was only observed in seven of 22 analyzed tows on Cruise 2. Cruise 2 was an anomaly relative to the other cruises, because the vessel targeted hard clams in addition to whelks and the captain wanted to demonstrate the spatial distribution and variability of benthic materials to the observer. Because *H. dianthus* and *S. vulgaris* only colonize hard substrates (Wells 1970, Curtis 1975, Tinsman & Hense 2004), worm-reef habitat is generally not dredged by the whelk fishery because of the difficulty of culling relatively few whelks from heavy nonliving material and potential damage to gear. Because most fishery effort and whelk catch comes from mud and sand habitats, it appears that the dredge fishery has a negligible effect on worm reef habitats. The cumulative effect of dredge fishery activity on mud and sand habitats, however, is unknown.

### Catch Composition

A variety of organisms were observed in whelk dredge samples and ratios of whelks to by-catch varied within and among observer cruises. Temporal, spatial, and gear variability presumably contributed to differences in catch composition. The highest variability on the proportion of knobbed whelks to by-catch was observed during Cruise 2 (Table 2), and is demonstrated by high proportions of hard clams and horseshoe crabs, and by fishing effort expended on a variety of bottom types, such as tube worm habitat, that held relatively few whelks. Ninety-three percent of all hard clams and 19% of horseshoe crabs enumerated in Delaware Bay samples came from Cruise 2. Variability in Cruise 2 catch composition occurred because hard clams were targeted in addition to whelks, and the captain chose to dredge a variety of bottom types. Proportions of knobbed whelks in samples were also low on cruises that were conducted in the Ocean. Lower numbers in volumetric samples from Cruises 8 and 11 may be explained by larger size of ocean whelks (Fig. 8) and by high levels of horseshoe crab by-catch. Taxa observed as by-catch in whelk trawl fisheries include Atlantic croaker *Micropogonias undulatus* (Linnaeus, 1766), sting

rays *Dasyatis* spp., horseshoe crabs, blue crabs and spotted seatrout *Cynoscion nebulosus* (Cuvier, 1830) in South Carolina (William Anderson; pers. comm.); and fringed flounder *Etropus crossotus* (Jordan & Gilbert, 1882), Atlantic stingray *Dasyatis sabina* (Lesueur, 1824), star drum *Stellifer lanceolatus* (Holbrook, 1855), southern stingray *Dasyatis americana* (Hildebrand & Schroeder, 1928), and southern kingfish *Menticirrhus americanus* (Linnaeus, 1758) in Georgia (James Page; pers. comm.).

The scarcity of channeled whelks in dredge samples is congruent with the observations of others that knobbed whelk dominate landings from dredge and trawl fisheries (Anderson et al. 1985, Davis & Sisson 1988) and channeled whelks dominate landings from pot or trap fisheries (Davis & Sisson 1988, Walker et al. 2003, Logothetis & Beresoff 2004). The absence of knobbed whelks in pots may be explained by a preference for live bivalves rather than bait and carrion, and potentially a reduced ability to enter traps because of a heavier shell than the thinner-shelled and more active channeled whelk (Davis 1981, Sisson & Wood 1988, Dobarro 1993, Walker et al. 2003, Logothetis & Beresoff 2004). Channel whelks have been documented as being less diurnally active than the knobbed whelk (Magalhaes 1948). During daylight, channel whelks may be sufficiently buried to be invulnerable to dredge gear.

### Size Distributions

Analysis of knobbed whelk size distributions from 2004 revealed that mean lengths were relatively similar among cruises in the Bay, but whelks from the Ocean were larger than those from the Bay. Delaware landings reports for 1994 to 2004 reported that only 3% of 3,031 dredging trips occurred in the Ocean. Log-book reports from 2004 indicate that 5% of 478 trips occurred in the Ocean. Lower levels of fishing effort and exploitation in the Ocean may explain spatial differences in size distributions. Observed differences between whelks collected in 2004 and whelks collected in 1993 to 1994, when there was no minimum legal length, suggest that the size distribution in the Bay has shifted to smaller individuals over that last decade. Length data from 2004 were recorded on-board prior to culling, whereas 1993 to 1994 data were recorded from landed whelks. Although there was no minimum legal length in 1993 to 1994, it is possible that smaller whelks were collected, but were culled and not landed. Regardless, the mode at 190 mm observed in the 1993 to 1994 distribution (Fig. 8) was absent in the 2004 length distribution. Increased exploitation (Fig. 2) may explain shifts in Bay size distributions over the last decade.

There is evidence that declines in annual whelk landings may result from over-harvest. Declines in catch and size of pot-caught channeled whelk in New England has been attributed to increased fishing pressure (Davis & Sisson 1988). The South Carolina trawl fishery experienced record landings in 1982 followed by precipitous declines in 1983 to 1984 (Fig. 9). Landings declines were attributed to high and sustained levels of fishing effort by South Carolina trawlers, coupled with low growth rates and confined home ranges of knobbed whelk determined from mark and recapture studies (Anderson et al. 1985). Presently, the United States South Atlantic whelk fishery is considered to be over-fished and overall annual landings are expected to remain low (Burrell 1997).

Landings from Delaware and New Jersey dredge fisheries are the highest on the Atlantic Coast (Figs. 2 and 9). In Delaware more whelks are landed from the Delaware Bay than from the Atlantic Ocean. Although whelks are also dredged from New Jersey's

coastal bays, a good portion of the whelks harvested by dredge that are landed in New Jersey come from the Delaware Bay (Jeffrey Normant; pers. comm.). Because of the magnitude of fishery activity in the Delaware Bay, it appears that whelk productivity in the Bay has been relatively high. The presence of sublegal whelks in commercial gear (Fig. 8) indicates that successful recruitment occurs. Despite increased fishery activity, whelk densities may remain elevated in some locations. For example, during Cruise 4, an estimated 3,677 legal whelks were taken from an approximately 14.2 ha area. If all 13 boats observed fishing the same bottom that day (Table 2) maintained an equivalent catch rate, the estimated density would be 3,354 legal whelk per ha or approximately 1 whelk per 3 m<sup>2</sup>.

Landings time series among the top-producing dredge and trawl fisheries in the Atlantic states reveal a general pattern of years of large landings followed by multiyear periods of smaller annual landings (Fig. 9). Delaware landings data (Fig. 2) suggest that the fishery is at a peak in the cycle as landings and effort have

been consistently high over the last four years relative to the 1994 to 2000 period. Demand for domestic whelk meats appears to be consistently high and is partially driven by depletion of global whelk fisheries (Kaplan & Boyer 1992); thus current effort levels in the Delaware fishery will continue until profitability declines. Given the experiences of other states and an apparent inability of whelk populations to sustain elevated exploitation for extended periods, the Delaware dredge fishery should expect reduced landings in the future.

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## EFFECTS OF DIETS, THEIR CONCENTRATIONS AND CLAM SIZE ON FILTRATION RATE OF HARD CLAMS (*MERETRIX LUSORIA*)

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**ABSTRACT** The hard clam (*Meretrix lusoria*) is one of the most important cultured molluscs in Taiwan. Although it is mainly cultured in ponds and supplemental feeding of artificial feed is commonly practiced, no scientific information on its preferred foods and appropriate feeding strategies are available. Six diets: fishmeal (F), soybean meal (S), commercial hard clam feed (C), microalgae *Tetraselmis chui* (T) and *Nannochloropsis oculata* (N) and bread yeast (Y), were fed to hard clams. Each diet was fed at 3 concentrations (20 mg L<sup>-1</sup>, 200 mg L<sup>-1</sup> and 633 mg L<sup>-1</sup>) and was fed to clams of 3 sizes (7–11 g, 12–17 g and 18–26 g total body weight) to gauge their effects on weight-specific filtration rate and pseudofeces production. Overall, the filtration rates for diets in descending order were S > C > F = Y > N > T. Particle size of the diets played an important, but not the only, role in determining the filtration rate. Diets with regular particle shape (N, T, Y) yield lower filtration rates and higher pseudofeces production than diets with irregular shape (C, F, S). Both filtration rate and pseudofeces production were higher for yeast than for algae (T, N). Filtration rate and pseudofeces production were also higher for the mixed component diet (C) than for single component diets (F, S). Filtration rate, but not pseudofeces production, was higher for the plant-based diet (S) than for fishmeal (F). Both diet concentration and clam size had inverse correlations with weight-specific filtration rate. The effects of all interactions between diets, diet concentrations, and clam sizes on filtration rates were significant, reflecting the complexity of feeding behavior of *Meretrix lusoria*.

**KEY WORDS:** clam, *Meretrix lusoria*, filtration rate, pseudofeces, commercial feed, microalgae, fishmeal, soybean meal

### INTRODUCTION

The hard clam (*Meretrix lusoria*) is one of the most important cultured molluscs in Taiwan. Its reported peak culture area was 7,552 ha in 1994, peak production was 31,517 t in 2003, peak productivity was 4.85 t ha<sup>-1</sup> in 2002 (Fig. 1). Hard clams were introduced to the northern coast of Taiwan from Japan in 1925 and later extensively ranched in sandy tidal flats, especially on the west coast. Before 1970, hard clams were cultured in ponds (Chen 1984) although it is unknown when this activity first started. In 1980, pond-culture accounted for only 13% of the total culture area. Hard clams were mostly cultured on sandy tidal flats. After mass artificial propagation was achieved (Chen & Lyuu 1982), total production increased 74% from 9,200 t in 1982 to 16,049 t in 1983 but also the importance of pond-culture. In 2003, around 83% of the culture area was in ponds and 94% of the production was from ponds.

Various artificial diets, either as supplements or as the main food source for larval, juvenile and adult bivalves have been developed, which include dried algae (Laing et al. 1990, Gladue 1991, Laing & Gil Verdugo 1991, Laing & Millican 1992), preserved algal pastes (Donaldson 1991), microencapsulated diets (Jones et al. 1984, Langdon et al. 1985) and yeast-based diets (Epifanio 1979, Urban & Langdon 1984, Coutteau et al. 1990, 1991). Such feeding, conducted either experimentally or commercially, however, was mostly done in hatcheries or nursery ponds (Southgate et al. 1998), not in growout ponds. In Taiwan, feed accounted for 14% to 37% of total production cost of pond-cultured hard clam (Guo 2003). There are two feed mills in Taiwan producing formulated hard clam feed, which is used by some farmers.

Hard clams have much higher productivity when cultured in ponds than when ranched in sandy tidal flats. In 2003, productivity in the former, 5,480 kg ha<sup>-1</sup>, was 2.2 times higher than that in the latter, 1,710 kg ha<sup>-1</sup> (Fisheries Administration 2003). In addition,

higher stocking densities are used in growout ponds: 1.00 to 1.60 million seed clams (0.8–1.0 g) ha<sup>-1</sup> versus <1.00 million seed clams ha<sup>-1</sup>, and faster growth occurs in ponds: 6–8 mo versus 1 year to reach marketable size (20 g total body wet weight) (Chen 1984). Organic fertilization with rice bran, chicken droppings, hog manure, and supplemental feeding contribute greatly to the food supply in ponds (Ho 1991). In Taiwan, food organisms used in hatcheries or nursery ponds include the algae *Isochrysis* sp., *Platymonas* sp., yeast and photosynthetic bacteria *Rhodospirillum* sp. Supplemental foods used for hard clam growout ponds include fishmeal, fish soluble, soybean meal, commercial formulated food and other home made mixtures in powder form. Clam farmers develop their diets and feeding strategies, such as the amount and timing of food delivery and assess the feeding effects on environmental quality completely based on their own experience, without using the available information from scientific studies. Therefore, this study aims to determine the effects of various concentrations of six diets that are currently or could potentially be used for hard clam aquaculture, on filtration rate and pseudofeces production of hard clams of different sizes so that this basic information can be used to develop compound feed formulation and appropriate feeding strategies.

### MATERIALS AND METHODS

#### *Experimental Variables: Diets, Their Concentrations and Clam Sizes*

The 6 experimental diets evaluated were fishmeal (F), soybean meal (S), commercial hard clam formulated feed (C), live microalgae: *Tetraselmis chui* (T) and *Nannochloropsis oculata* (N) and bread yeast (Y). The major ingredients in C are S, F, Y, squid meal, corn meal, oyster shell, yeast and calcium perphosphate. The algae T and N were often found in hard clam ponds during our field observations and have been widely used as feed for bivalves (Laing & Gil Verdugo 1991, Laing & Millican 1992). The feed components F, S, Y and C were obtained from a commercial source (Taishan Co., Salu, Taichuan, Taiwan). Microalgal stock cultures were obtained from Biotechnology Section, Taiwan Fish-

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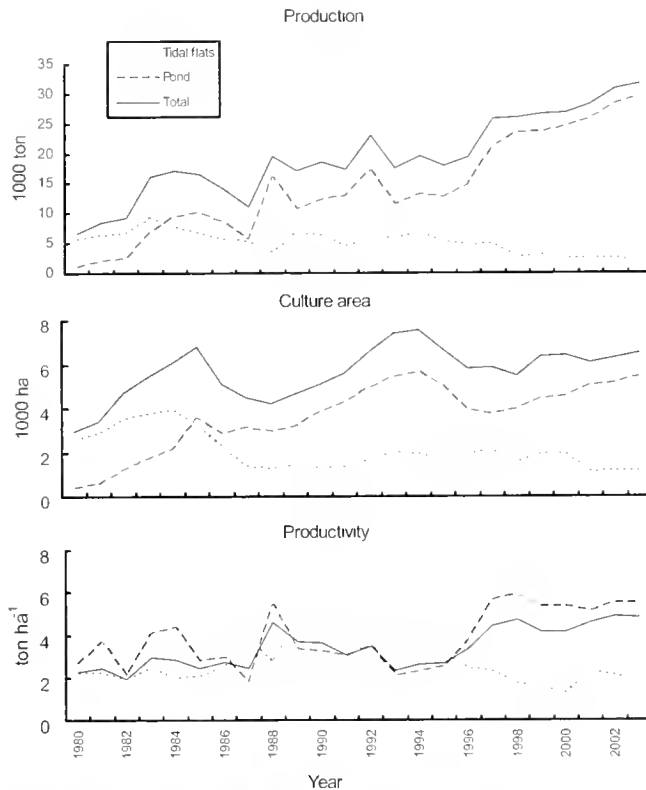


Figure 1. Production statistics of hard clams *Meretrix lusoria*, in Taiwan between 1980 and 2003.

eries Research Institute. These algae were cultured in 5-L glass flasks containing Provasoli's enriched seawater (35‰ salinity, 0.45  $\mu\text{m}$  filtered and UV treated) (Provasoli 1968). Cultures were held at 25°C under cool white fluorescent light on a 12 hL:12 hD photoperiod. Algae were used during mid stationary phase of growth. The diet F and S were further ground by a locally made feed ingredient grinder for 4 min and 8 min, respectively to obtain finer particles sizes. Particle sizes were determined by measuring at least 300 diet particles to the nearest 5  $\mu\text{m}$  with a hemacytometer under a microscope (Nikon E400, Tokyo, Japan). Intervals were set: <5  $\mu\text{m}$ , every 5  $\mu\text{m}$  from 6–30  $\mu\text{m}$  and >30  $\mu\text{m}$ . Particle sizes (mean maximum length  $\pm$  standard deviation) and shape (determined qualitatively) of the experimental diets were: F = 17.3  $\pm$  7.5  $\mu\text{m}$ , irregular; S = 19.5  $\pm$  10.2  $\mu\text{m}$ , irregular; C = 12.5  $\pm$  6.3  $\mu\text{m}$ , irregular; T = 8.0  $\pm$  1.5  $\mu\text{m}$ , oval; n = 4.1  $\pm$  0.7  $\mu\text{m}$ , round and Y = 6.2  $\pm$  1.0  $\mu\text{m}$ , round.

The diet concentrations used were determined by using the maximum concentration of *N. oculata* that could be cultured in this laboratory, around 2,000 mg dry biomass  $\text{L}^{-1}$ , as 2 units in a  $\log_{10}$  scale. We then chose 0.5, 1 and 1.5 units as the concentrations for use, which were equivalent to 20 mg, 200 mg and 633 mg dry weight  $\text{L}^{-1}$ . In clam ponds studied, algal concentration could reach as much as 435 mg dry weight  $\text{L}^{-1}$  depending on their productivity.

Dry weights of algal cells of both species were determined by filtering algae from a 100-mL aliquot of suspension of known concentration; aliquots were taken from five replicate cultures of each species. Algae were retained on tared, glass-fiber filters (Whatman, no. GF/F, 0.7- $\mu\text{m}$  pore size), which were subsequently washed with a 0.5 M solution of ammonium formate to remove sea salts. Filters were then dried at 100°C for 2 h to volatilize the

ammonium formate (Epifanio & Ewart 1977) and weighed on an analytical balance to a precision of 0.1 mg. Algal density in dry weight base were 1.18 ( $\pm 0.43$ )  $\times 10^8$  cells  $\text{mg}^{-1}$  and 1.85 ( $\pm 0.67$ )  $\times 10^7$  cells  $\text{mg}^{-1}$  for N and T, respectively.

Three size classes of clam were used: small, 7–11.9 g (total body wet weight); medium, 12–17.9 g and large, 18–26 g. Clams were obtained from ponds in Taishi station, Mariculture Research Center, Taiwan Fisheries Research Institute. At our laboratory, they were acclimated for a week under ambient temperature in a 2,000-L fabricated reinforced polyethylene (FRP) round tank paved with 6–10 cm sand, and fed a combination of all 6 diets on an equal dry weight basis. Salinity was maintained at 15‰ to 18‰, which was the salinity in the ponds where the clams were from. One day before experimentation, an adequate number of clams were collected and placed in a 20-L bucket filled with 1- $\mu\text{m}$  filtered seawater. Water temperature was controlled at around 25.2  $\pm$  0.4 °C and clams were not fed.

#### Standard Curve of Suspended Particles

A turbidity-weight relationship was developed to estimate the biomass of each diet in the water. Five concentrations: 20 mg, 63.3 mg, 200 mg, 633 mg and 2,000 mg  $\text{L}^{-1}$  of F, S, C and Y suspension were obtained by weighing 0.012, 0.038, 0.12, 0.38 and 1.2 g of each diet into a beaker containing 600 mL filtered seawater and suspended with a magnetic stirrer. A 10-mL sample was taken by pipette and immediately measured in a turbidity meter (HACH 2100P, Loveland, Colorado, USA). For each concentration, 5 replicates were measured and for each replicate, 2 repeated readings were obtained. For the microalgae, instead of dilution, the original algal suspension was concentrated several times by centrifugation at  $\times 3,000$  rpm for 10 min each time to obtain a calibration.

#### Filtration Rate Apparatus

Clearance rates were determined using methods similar to those of Shumway et al. (1985) and Levinton et al. (2002). Filtration rate was determined by the indirect method (Epifanio & Ewart 1977, Winter 1978), measuring the removal of suspended particles from a known volume of water per unit time (Fox et al. 1937). This "indirect method" agrees well with other indirect and direct methods used for measuring filtration rates in mussels (Famme et al. 1986). The feeding apparatus was composed of a 1-L beaker held over a magnetic stirrer (MS-90, Fargo). The beaker contained a feeding platform, set above a 3-cm Teflon stirrer, made by tying three 3.5-cm rods perpendicularly to a 6-cm ring. Both ring and rods were made of no. 19 gauge (1.9 mm inner diameter) insulated wire. The ring was covered with a 2 mm  $\times$  2 mm mesh nylon net. The dial of the stirrer was fixed at 3.5 so that mixing kept all diet particles in suspension but would not resuspend the clam's fecal material. One clam was used per beaker.

Each trial was conducted as follows: a designated diet suspension was prepared and 600 mL of diet suspension was poured into a 1-L beaker. The stirrer was activated and a designated size clam, which had been starved for 24 h, was placed on the center of the feeding platform. A 10-mL sample of suspension was collected to measure the initial diet concentration. The experiment ran for 1 h starting when the clam resumed feeding and extended and dilated its siphon. Another sample was taken at the end of the hour for the final diet concentration. In a few preliminary trials, when large size clams were used to test their filtration capability in diet F and S at a concentration 20 mg  $\text{L}^{-1}$  for 10 and 15 min, no significant



reduction in diet concentration were found. In a similar clearance experiment, Levinton et al. (2002) used a clearance period of 0.75 h for mussels and 1.5 h for oysters, thus we chose 1 h for each trial. Experimental bivalves were then opened; their soft tissue was separated from the shell, blotted with a paper towel and then dried at 105°C for around 48 h until a constant weight was reached. Dry weight data were used to calculate weight specific filtration rate.

Considering the deviation of experimental conditions from the natural ones, such as starvation prior to the measurement, constant circular flow around the clam and clam's laying position on the feeding platform, filtration rate determined by this indirect method may not be very representative of the true filtration rate of this hard clam in natural conditions. However, for the simplicity of our method, apparatus set-up and the consistency in use, the filtration rate should potentially be used in a comparative manner among treatments.

#### Filtration Rate Experiment

A  $3 \times 6 \times 3$  factorial arrangement treatments design was used in which three sizes of clam were exposed to six diets each at three concentrations. Each treatment combination had four replicates. Each replicate used 3 feeding chambers: a blank chamber using a dummy clam and two treatment chambers with live clams. The dummy clam consisted of an empty clam filled with sand with the shells glued together. In each replicate the clams had a similar shell size. The experiments in three chambers of a replicate were run simultaneously. After correcting by turbidity changes in the blank chamber, the two readings for live clams were averaged and used as one datum for that replicate.

The equation by Riisgård (1988) was used to estimate the filtration rate (F):

$$F = V/t \times \ln Co/Ct$$

where V is a known volume of water; t the time in h; ln the natural logarithm; and Co and Ct the particle concentrations at time 0 and t, respectively. Because the rate of filtration of bivalves is related to the size of the animal (Winter 1978) and clam weight varied for each trial, weight-specific filtration rate ( $FR = F/W$  in  $\text{mL h}^{-1} \text{g}^{-1}$ ) (Epifanio & Ewart 1977) was used: where W was dry weight of soft tissue mass (g) (Bayne et al. 1976).

#### Pseudofeces Production Experiment

A factorial treatments design: diets  $6 \times 2$  clam sizes were used in which each treatment had four replicates. Only 1 diet concentration was used,  $200 \text{ mg L}^{-1}$ . This was because some of the clams would not feed at  $633 \text{ mg L}^{-1}$ . On the other hand, at the lowest concentration,  $20 \text{ mg L}^{-1}$ , insufficient pseudofeces could be collected to allow accurate measurement. No medium size clams were available when conducting the experiment so only small and large clams were used.

After feeding for 1 h, the clam was removed from the beaker. A pipette was used to carefully collect the pseudofeces on the bottom or attached to the wall of the beaker or that clung to the feeding platform. They were placed in an aluminum foil plate (5 cm in diameter and about 1.9 g weight), dried to constant weight at 80°C and weighed to the nearest 0.01 mg.

#### Statistical Analysis

For filtration rate, a 3-way ANOVA was used to test the significance of the main effects and interactions of diets, diet con-

centrations and clam sizes. For pseudofeces production, a 2-way ANOVA was used to test the significance on the main effects and interactions of diets and clam sizes. Duncan's multiple range tests (DMRT) were used to test the differences among the levels of each main effect. Besides DMRT for pairing (one-to-one) comparisons, 5 orthogonal contrasts were conducted for systematic (set-to-set) comparisons: (1) diet particle shape: regular (N, T and Y) versus irregular (C, F and S); (2) regular particle shape: algae (N and T) versus yeast (Y); (3) algae: N versus T; (4) irregular particle shape: mixture components (C) versus single component (F and S) and (5) single component: plant material (S) versus animal material (F).

## RESULTS

#### Filtration Rate

There were highly significant ( $P \leq 0.01$ ) effects of diet, diet concentrations and clam sizes on filtration rate, and all the interactions were highly significant (Table 1). Within diet, orthogonal contrasts (Table 1) indicated that the average filtration rate for diets having regular shape of particles (i.e., N, T and Y) was significantly lower than that for diets having an irregular particle shape (i.e., C, F and S). For the regular particle shape diets, average filtration rate for the planktonic algae N and T was significantly lower than that for the yeast, Y. Between the two algae, clams fed N had significantly higher filtration rates than those fed T. For the irregular particle shape diets, filtration rate for the diet having mixed components (i.e., C) was significantly lower than the average filtration rate for diets having a single component F and S. Between the diets that had a single component, clam filtration rate of S was significantly higher than that of F. Disregarding clam sizes, DMRT results showed that the filtration rates in descending order were  $S > C > F = Y > N > T$  (Fig. 2). For small clams, all differences in filtration rate among diets were significant (i.e.,  $S > C > F > Y > N > T$ ). However, for larger clams, the differences in filtration rate among diets became less distinct. For example, for large clams, there were no differences in filtration rate between S and F, and also no differences among S, Y and C.

Overall, filtration rate decreased with increasing diet concentration (Fig. 3). Such an inverse relationship was especially evident between diet concentration  $633 \text{ mg L}^{-1}$  and  $200 \text{ mg L}^{-1}$  and this effect was consistent for each clam size class (i.e., whereas the former diet concentration was about 3 times as the latter, the filtration rate at the former was about 1/3 of the latter).

Disregarding diet and diet concentration, the smaller the clam, the higher the weight-specific filtration rate. The filtration rates differed significantly and were  $49 \pm 2$ ,  $30 \pm 1$  and  $27 \pm 1 \text{ mL h}^{-1} \text{g}^{-1}$  for small, medium and large clams, respectively.

#### Pseudofeces Production

There were highly significant effects of diet and clam size on pseudofeces production. No interaction was found between diet and clam size on pseudofeces production (Table 2). Overall, large clams produced significantly less pseudofeces ( $1.3 \pm 0.9 \text{ mg g}^{-1}$ ) than small clams ( $1.7 \pm 0.8 \text{ mg g}^{-1}$ ).

Within the diet effects, orthogonal contrasts (Table 2) indicated that the average pseudofeces production from diets having a regular particle shape (i.e., N, T and Y) did not differ from that of diets having an irregular particle shape (i.e., C, F and S). For the diet with a regular particle shape, average pseudofeces production from

TABLE 1.

Main effects of 6 diets, 3 diet concentrations, 3 clam sizes, their interactions and 5 orthogonal contrasts for diets on filtration rate of hard clam.

SV	Mean	df	SS	MS	F	P > F
Model		53	125,313	2,364	69	<0.01
Diet		5	13,948	2,789	81	<0.01
Shape (Reg. vs. Irreg.)						
(N, T, Y vs. C, F, S)	29 vs. 42	1	8,930	8,930	16.04	<0.01
Reg. (Algae vs. Yeast)						
(N, T vs. Y)	26 vs. 36	1	2,280	2,280	4.10	<0.01
Algae (Nano. vs. Tetra.)						
(N vs. T)	27 vs. 24	1	137	137	0.25	<0.05
Irreg. (Mixture vs. Single)						
(C vs. F, S)	40 vs. 43	1	173	173	0.31	<0.05
Single (Soy. vs. Fish.)						
(S vs. F)	49 vs. 37	1	2,428	2,428	4.36	<0.01
Clam size		2	20,376	10,188	298	<0.01
Clam size × Diet		10	5,402	540	16	<0.01
Conc.		2	51,127	25,563	748	<0.01
Clam size × Conc.		4	2,074	3,018	88	<0.01
Diet × Conc.		10	11,689	1,169	34	<0.01
Clam size × Diet × Conc.		20	10,697	535	16	<0.01
Error		162	5,538	34		
Total		215	130,851			

the two planktonic algae N and T, was significantly lower than that from the yeast Y. Clams fed on N produced significantly less pseudofeces than those fed on T. For the diet with irregular particle shape, pseudofeces production from the mixed diet (i.e., C) was significantly lower than that from single-component diets (i.e., F and S). Between the single-component diets, the pseudofeces production from S was not different from that of F. Among-diet effects showed that for clams of both sizes (Fig. 4), those fed on Y produced the highest amount of pseudofeces. Among the remaining diets, pseudofeces production descended in the following order:  $S \geq F \geq T \geq C \geq N$ . Pseudofeces production for F, T and C did not differ significantly. When DMRT was performed by clam size separately, the order for large or small size classes remained the same as for both sizes combined. The only difference in DMRT results between large and small clams was that for the latter Y was not significantly greater than S but for the former pseudofeces production differed significantly between these two diets.

#### DISCUSSION

In general, suspension-feeding bivalve molluscs have adopted several strategies for controlling the ingestion of particulate matter (MacDonald & Ward 1994), including regulation of (1) feeding duration (Foster-Smith 1975); (2) clearance rates (Bayne & Newell 1983) and (3) pseudofeces production (Kiorboe et al. 1980, Newell & Jordan 1983). Various criteria are often proposed as the basis for particle selection including physical features: such as particle size (Tammes & Dral 1955, Möhlenberg & Riisgård 1978, Riisgård 1988, Langdon & Newell 1990), shape (Bayne et al. 1977), aggregation (Waite et al. 1995), motility and density (Brilliant & MacDonald 2000), chemical cues: such as energy content, C/N ratio (Ward & MacDonald 1996), organic content (Bacon et al. 1998, Defossez & Hawkins 1997) and chlorophyll content (Nakamura 2001). The production of pseudofeces can serve to improve the quality of material ingested by means of selective rejection, rather than simply the elimination of excess material of the ingestive capacity (MacDonald & Ward 1994). Selective rejection

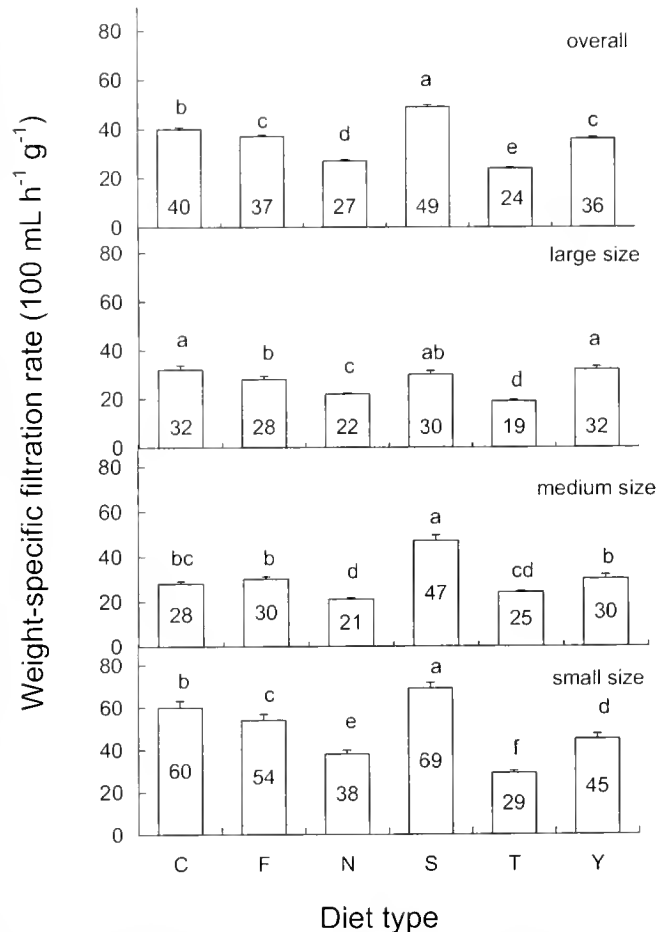


Figure 2. Average filtration rate of 3 sizes of hard clams receiving 6 diets (C; commercial hard clam feed, F; fishmeal, N; microalgae *Nannochloropsis oculata*, S; soybean meal, T; microalgae *Tetraselmis chui*, and Y; bread yeast). Averages not sharing any common alphabet (above data column) are significantly ( $P \leq 0.05$ ) different.

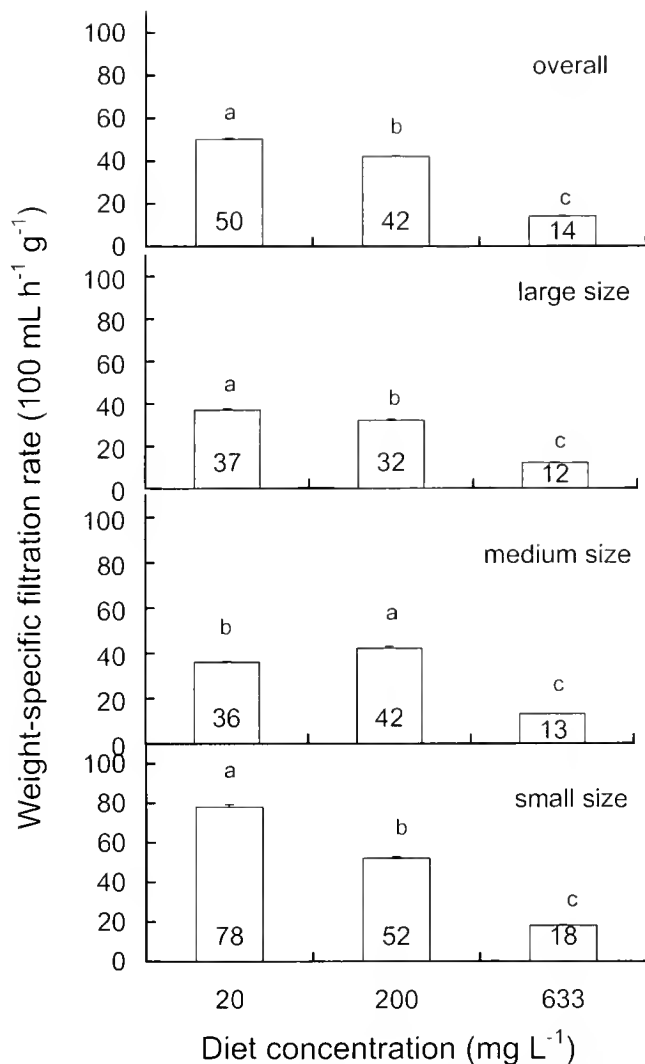


Figure 3. Average filtration rate of 3 sizes of hard clams receiving 6 diets at 3 concentrations. Averages not sharing any common alphabet (above data column) are significantly ( $P \leq 0.05$ ) different.

mainly involves preferential rejection of nonchlorophyll-containing particles (Kiorbøe & Møhlenberg 1981, Newell & Jordan 1983) and reduction of the proportion of particulate inorganic matter in pseudofeces (Widdows et al. 1979, Kiorbøe et al. 1980, Kiorbøe & Møhlenberg 1981, Bricelj & Malouf 1984, Defossez & Hawkins 1997).

#### Diets

The highest filtration rate obtained for soybean meal in this study (Fig. 2) could be mainly attributed to its having the largest particle size, which made S easier to be retained by the gill of clam and left less particles in water. Previous studies have demonstrated the importance of particle size in mediating selection processes in some bivalve species (Defossez & Daguzan 1996, Defossez & Hawkins 1997). Wisely and Reid (1978) considered the selection of feed by members of the order Eulamellibranchia (oysters, cockles and clams); selection appeared to be made on the basis of particle size regardless of potential food value. In *Mytilus edulis*, *Cerastoderma edule* and *Venerupis pullastra*, the rates of ingestion of particles were found to be roughly proportional to the size of the particles (Foster-Smith 1975). A few studies indicated that bi-

valves had higher filtration or retention efficiency for larger particles. The filtration efficiency of bacterioplankton (0.2–2  $\mu\text{m}$ ) by *Geukensia demissa* was 42% lower than that of phytoplankton >2  $\mu\text{m}$  (Wright et al. 1982). *Mercenaria mercenaria* completely retained particles above 4  $\mu\text{m}$ . Below this size threshold retention efficiency gradually decreased to between 35% and 70% for 2- $\mu\text{m}$  particles (Riisgaard 1988). In the clam *Ruditapes decussatus* particles smaller than 3  $\mu\text{m}$  in diameter, which include bacteria and clay particles, were retained with low efficiency (i.e., <75%). Algal cells, such as phytoplankton and other particles in the size range 3–8  $\mu\text{m}$  were efficiently retained (70% to 100% retention) by the clam (Sobral & Widdows 2000). The studies mentioned earlier concluded that filtration retention efficiency increased with increasing particle size from 0.2–8  $\mu\text{m}$ . In our study, because the particle size of diets varied over a wider range, 4  $\mu\text{m}$  to 19  $\mu\text{m}$ , the overall ranking of filtration rates for the diets ( $S > C > F = Y > N > T$ , Fig. 2) did not completely follow the ranking of particle size:  $S$  (19  $\mu\text{m}$ ) >  $F$  (17  $\mu\text{m}$ ) >  $C$  (12  $\mu\text{m}$ ) >  $T$  (8  $\mu\text{m}$ ) >  $Y$  (6  $\mu\text{m}$ ) >  $N$  (4  $\mu\text{m}$ ). Bacon et al. (1998) indicated that because most of the particles used in their study were between 4 and 6  $\mu\text{m}$  in diameter, size-dependent selection was probably not a significant factor and selection by the softshell clam *M. arenaria* was based on organic content, or perhaps other qualitative particle characteristics.

That particle size that can affect pseudofeces production is indicated by the comparison of the two algae T and N; the higher pseudofeces production when clams were fed on T may be attributed to the fact that T particles are twice as big as N particles or to other confounding factor. Similarly, Defossez and Hawkins (1997) experimented with the mussel *Mytilus edulis* and clams *Ruditapes philippinarum* and *Tapes decussatus* and they concluded that particles with diameters larger than from between 7.5 and 22.5  $\mu\text{m}$  were preferentially rejected as pseudofeces. As with filtration rate, particle size was not the only factor affecting pseudofeces production. In our study, pseudofeces production in descending order was  $Y > S \geq F \geq T \geq C \geq N$  (Fig. 4), which again did not follow completely the order of particle size. The result that particle selection via pseudofeces production was not totally based on the size, was in agreement with MacDonald and Ward (1994).

The results of the orthogonal contrast indicated that the average filtration rate for diets with a more regular particle shape (i.e., N, T and Y) was significantly lower than that for diets consisting of irregular particle shape (i.e., C, F and S). Few studies have been published on the effects of shape regularity of diet particles on filtration rate in bivalves. Bayne et al. (1977) pointed out that elongated or triradiated algal cells might be more efficiently retained than spherical particles of the same volume. Wisely and Reid (1978) mentioned that rice particles that were used in their study, when viewed microscopically, were mainly irregularly shaped but many of them had a characteristic "hexagonal" appearance. Irregular shape may not only extend particle size in one dimension but the rough extrusions may also favor the retention of particles by the gill and consequently reduce the particle density in water in this study.

Limited studies showed that a diet of 50/50 mixture of algae and yeast supported growth comparable to a 100% algal ration when fed to the hard clam *Mercenaria mercenaria* (Epifanio 1979) and oyster *Crassostrea virginica* (Alatalo 1980). Although yeast could be used as algal substitutes for feeding bivalves, filtration rate or ingestion selectivity was not compared between yeast and algae in those studies. In our study, filtration rate and pseudofeces production were higher for yeast Y than for planktonic algae N and T grouped together. The higher filtration rate for Y could not be

TABLE 2.

Main effects of 6 diets, 2 clam sizes, their interactions and 5 orthogonal contrasts for diets on hard clam pseudofeces production.

SV	Mean	df	SS	MS	F	P > F
Model		11	27.48	2.50	15.04	<0.01
Diet		5	24.69	4.94	29.73	<0.01
Shape (Reg. vs. Irreg.)						
(N, T, Y vs. C, F, S)	1.42 vs. 1.39	1	0.49	0.49	2.96	0.09
Reg. (Algae vs. Yeast)						
(N, T vs. Y)	0.95 vs. 2.37	1	20.15	20.15	121.34	<0.01
Algae (Nano. vs. Tetra.)						
(N vs. T)	0.73 vs. 1.17	1	0.79	0.79	4.75	0.04
Irreg. (Mixture vs. Single)						
(C vs. F, S)	0.90 vs. 1.65	1	2.97	2.97	17.89	<0.01
Single (Soy. vs. Fish.)						
(S vs. F)	1.78 vs. 1.51	1	0.28	0.28	1.69	0.20
Clam size		1	1.49	1.49	9.00	<0.01
Clam size × Diet		5	1.30	0.26	1.57	0.19
Error		36	5.98	0.17		
Corrected Total		47	33.46			

attributed to particle size, because particle size of Y (6  $\mu\text{m}$ ) was between T (8  $\mu\text{m}$ ) and N (4  $\mu\text{m}$ ). Nonetheless particles above 4  $\mu\text{m}$  can be completely retained by *Mercenaria mercenaria* (Riisgård 1988). Higher pseudofeces production in Y for this hard clam might be attributed to yeast's lack of chlorophyll, because it was suggested that one of the two ways in which scallops could improve the quality of material ingested was through preferential rejection of nonchlorophyll-containing particles in the pseudofeces (Kiorboe & Møhlenberg 1981, Newell & Jordan 1983).

Filtration rates of clams fed on C were higher than when they were fed on F, Y and N, which were larger in particle size. This could be attributed to the hard clam's greater acceptance of C, which is possibly closer to a natural suspension comprising a mixture of various components than the other diets that contain only a single component. Previous studies demonstrated that addition of a small amount of mud to an algal suspension made the suspension closer to a natural one and only improved the clearance rate for *Mytilus edulis* (Kiorboe et al. 1980, Bayne et al. 1987) and *V. corrugatus* (Stenton-Dozey & Brown 1994).

Bivalves are traditionally regarded as suspension feeders for mainly phytoplankton and organic particles. In our study, pseudofeces production for F was no greater than S suggesting that for this clam preference for fishmeal, an animal-based substance, was no less than soybean meal, a plant-based substance. The higher filtration rate for S than for F could be attributed to its being 2  $\mu\text{m}$  larger than the latter, or better quality.

#### Diet Concentrations

Our findings that filtration rate in *Meretrix lusoria* decreased with increasing concentration for all diets (Fig. 4) were in agreement with other literature studies for *Mytilus edulis*, *Cerastoderma edule* and *Venerupis pullastra*, filtering purely algal suspensions (Foster-Smith 1975); for *Mytilus edulis* feeding on resuspended fine mud (Widdows et al. 1979); for *Ostrea edulis* (Grant et al. 1990), for *Mya arenaria* (Grant & Thorpe 1991) and for *Cerastoderma edule* (Iglesias et al. 1992, Navarro & Widdows 1997), all feeding on mixtures of algal cells and suspended silt; and for *Ruditapes decussatus*, filtering suspended particulate matter (Sobral & Widdows 2000).

Bivalves can typically maintain a constant ingestion when ex-

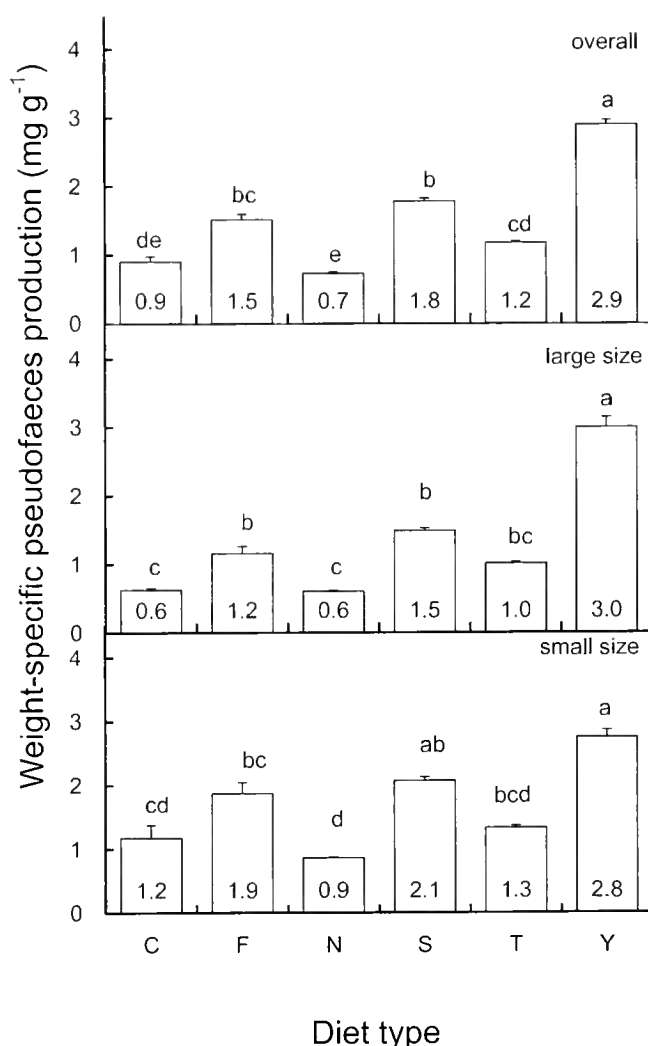


Figure 4. Pseudofeces production of 2 size classes of hard clams receiving 6 diets (C; commercial hard clam feed, F; fishmeal, N; microalgae *Nannochloropsis oculata*, S; soybean meal, T; microalgae *Tetraselmis chui*, and Y; bread yeast). Averages not sharing any common alphabet (above data column) are significantly ( $P \leq 0.05$ ) different.

posed to increasing seston concentrations by reducing clearance rate, increasing pseudofeces production or some combination of both mechanisms (Foster-Smith 1975, Winter 1978, Kiorboe et al. 1980, Bricelj & Malouf 1984, Bacon et al. 1998).

Filter-feeding activity is a function of cell concentration, which has been well documented by several authors for different species of lamellibranchiate bivalves (Sania 1976, Epifanio & Ewart 1977, Winter 1978). Similar reductions in clearance rates in response to increasing particle concentrations have been observed in epifaunal bivalves (Bayne et al. 1987) and infaunal bivalves including *Mercenaria mercenaria* (Bricelj & Malouf 1984). *Mya arenaria* significantly decreases its clearance rate in response to increasing particle concentration, showing about a 50% decline in rates, especially between 1 and 7 mg L<sup>-1</sup> (Bacon et al. 1998). In our study, although diet concentrations were much higher, ranging between 20 mg L<sup>-1</sup> and 633 mg L<sup>-1</sup>, the decrease in filtration rate still held.

#### Clam Sizes

There have been few studies on body size effects on filtration rate in tropical marine bivalves, such as the species used in this study. Our results showed that weight-specific filtration rate declined with increase in body size (Fig. 1, Fig. 2). Instead of using weight-specific filtration rate, Riisgård (1988) demonstrated that the filtration rate ( $F$ , L h<sup>-1</sup>) in *Mercenaria mercenaria*, *Crassostrea virginica* and *Geukensia demissa* increased with increasing dry weight of soft parts ( $W$ , g) according to the equation:  $F = aW^b$ . Such allometric relationship was well applied to several bivalves of which the filtration rates were measured by various methods (Gosling 2003). Nakamura (2001) also showed that the clearance rate for each category of chlorophyll *a*-containing particles (bacteria, picocyanobacteria, flagellates and *Nitzschia*) had a positive correlation with soft-body dry weight. In fact, there was no contradiction in results between those and ours, because weight-specific filtration rate or unit weight filtration rate (Epifanio & Ewart 1977) was inversely related to the weight of the animal. That the rates of physiological processes increase as power

relationships with increasing body size, but rates per unit body mass tend to decrease with increasing size (Yukihira et al. 1998) provided further explanation for the relationship between filtration rate or weight-specific filtration rate and weight. Disregarding diet type, diet concentration and size class the allometric relationship between filtration rate and weight in this study was  $F = 6.95 W^{0.81}$ .

#### Interaction

This study showed that the interactions between clam sizes, diets and diet concentrations had significant effects on filtration rate. It was obvious that the acceptance of various diets by small clams was different from that in large clams, if filtration rate was used as a measure of diet acceptance. Such interaction effects on filtration rate, to our surprise, were almost never reported. The only related study by Pérez-Camacho et al. (1994), indicated that the filtration rate of *Ruditapes decussatus* veliger larvae decreased with increasing diet concentration; however, the decrease in filtration rate was less pronounced in large than small clams. Such dissimilar responses for animals of different sizes were similar to those obtained in our study.

This study used filtration rate and pseudofeces production as response parameters to determine the ingestion preference of various sizes of hard clams for diets of various categories and concentrations. We first used DMRT to examine the feasibility of using particle size as an individual effect to explain ingestion preference across all 6 diets. Furthermore, we used orthogonal contrasts to compare systematically the ingestion preference for diets under various categories, such as shape regularity of diet particle (regular vs. irregular), chlorophyll content in live microbes (phytoplankton vs. yeast), complexity of diet composition (single component vs. multiple components) and plant-based diet versus fishmeal. The results suggested the complications in ingestion preference (Ward & Shumway 2004) require many more specific and detailed trials before a practical and realistic compound feed for the hard clam can be formulated.

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## GROWTH RATE AND LONGEVITY OF *DREISSENA POLYMORPHA* (PALLAS): A REVIEW AND RECOMMENDATIONS FOR FUTURE STUDY

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**ABSTRACT** We review the variety of methods that have been used over the last 50 y in the Former Soviet Union, Eastern and Western Europe, and recently in North America to determine growth rate and longevity in zebra mussels (*Dreissena polymorpha* [Pallas]). These methods include: counting annual rings, analysis of size-frequency distributions, following growth under experimental conditions and monitoring marked mussels under natural conditions, without removing them from substrate. The last method provides the most reliable data, however this is the least common method used. *Dreissena polymorpha* growth rates depend on water temperature, season of the year, location in the water column, food availability, oxygen concentrations, water velocity and various other environmental factors. However, it is very difficult to separate the independent effects of each of these factors, especially in natural waterbodies. Several factors may overlap and have additive or synergistic effect that makes it difficult to determine the effects of a single factor. When comparing among studies that used the same methods, we found that zebra mussels grow faster in reservoirs than in lakes. The reported longevity of *D. polymorpha* varies from 2–19 y and it is not clear to what extent this variation is caused by biological variability and environmental conditions and what amount of the variation is caused by the methods used to assess age and longevity.

**KEY WORDS:** zebra mussels, *Dreissena polymorpha*, growth, growth rate, methods, longevity

### INTRODUCTION

The zebra mussel, *Dreissena polymorpha* (Pallas), is one of the most pervasive invaders in freshwaters of the northern hemisphere. However, many aspects of the basic biology of *D. polymorpha* that are necessary for understanding and predicting the population dynamics and ecological impacts of this invader are still not well known. In addition, much of the research on the biology of *D. polymorpha* that has been conducted in the former Soviet Union (FSU), has not been published in English, and therefore it is not available to most scientists currently studying *D. polymorpha*. Growth rate and longevity are particularly important for understanding the population biology and ecological impacts of zebra mussels, especially because fecundity and filtering capacity increase with body size. Most of the published research has been conducted with the invasive subspecies *Dreissena polymorpha polymorpha*, which is capable of living in totally fresh water and has been the major invader in most places where dreissenids have been introduced. Less work has been conducted with *Dreissena bugensis* (Zhuravel 1951, MacIsaac 1994, Baldwin et al. 2002), *Dreissena polymorpha andrusovi* (Karpevich 1952, 1964, Lvova et al. 1983, 1994) and *Dreissena caspia* (Karpevich 1952, 1964).

Here we review the variety of methods used to estimate growth and longevity of zebra mussels over the last 50 y (Table 1, Table 2), discuss limitations of each and recommend the most appropriate methods for measuring growth and longevity in the field. We also synthesize the impacts of a range of environmental factors on growth and longevity in zebra mussels.

### METHODS TO ESTIMATE GROWTH RATE

#### Rings on Shells

One of the oldest and most common methods for estimating the growth rate of zebra mussels is by counting annual rings on shells

of different sizes, and then calculating the average length of each age group of *Dreissena* in a population (Karpevich 1952, 1964, Kachanova 1963, Stanczykowska 1964, Lyakhov & Mikheev 1964, Mikheev 1964, Kornobis 1977, Karatayev & Tishchikov 1979, Kirpichenko & Antonov 1982, Dorgelo & Gorter 1984, Draulans & Wouters 1988, Miroshnichenko 1990). Plotting the average size of each age group against their age provides a growth rate curve. The advantage of this method is that by measuring individuals at a single point in time estimates of growth over several years can be made. However, counting growth rings is very subjective as it is difficult to distinguish annual rings from rings formed because of other factors that slow growth. Morton (1969a) found that two rings are formed annually: when growth slows during the winter and during spawning. Lvova (1980) found in the Uchinskoe Reservoir, 3–9 rings on the shells of 3-y-old mussels grown in cages for 2 y. In Czos Lake Lewandowski (1983) found from 1–3 rings on the shells of 1-y-old *D. polymorpha*, and from 2–5 rings on 2-y-old mussels.

Many other authors have also reported difficulties in distinguishing annual rings (Karpevich 1964, Kirpichenko 1965, Morton 1969a, Wiktor 1969, Lvova-Kachanova 1972, Lvova 1980, Lewandowski 1982a, Karatayev 1983, bij de Vaate 1991, Lvova et al. 1994). Often mussels with distinct rings can be found side by side with mussels without rings (Lvova 1980, Lvova et al. 1994, Jantz 1996). Moreover, 1-mm zebra mussels that settle at the end of the growing season do not produce a first annual ring. Therefore, these mussels would be incorrectly identified as young-of-the-year the following year.

#### Size-Frequency Distributions

Size-frequency distributions have been used in a number of studies of *D. polymorpha* growth rates (Morton 1969a, Jantz & Neumann 1992, Martel 1993, 1995, Smit et al. 1993, Dall & Hamburger 1996, Chase & Bailey 1999a, Orlova & Panov 2004), and can be useful if there is highly synchronized spawning and settlement and low interindividual variation in growth. Newly settled

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TABLE 1.  
Impact of environmental factors on the growth rate of *Dreissena polymorpha*.

Factor	Impact	References
Temperature increase	Accelerates growth if maximum temperature <30 °C	Mikheev 1964, Morton 1969a, Spiridonov 1969, Yaroshenko & Naberezhnyi 1971, Lvova-Kachanova 1972, Skalskaya 1976a, 1976b, Stanczykowska 1976a, Kornobis 1977, Elagina et al. 1978, Walz 1978b, 1978c, Karatayev & Tishchikov 1979, Lvova 1980, Karatayev 1983, 1984, 1988, Smit et al. 1992, 1993, MacIsaac 1994, Jantz 1996
Season of a year	Maximum growth is usually at the beginning of the growing season	Mikheev 1964, Lvova 1980, Karatayev 1983, Sprung 1995a, Jantz 1996, Burlakova 1998
Location in the water column	Growth is faster in the water column than on the bottom	Kachanova 1963, Spiridonov 1971, Kornobis 1977, bij de Vaate 1991, Yu & Culver 1999
Trophic conditions	Growth is faster in eutrophic than oligotrophic waters	Walz 1978a, Dorgelo & Gorter 1984, Smit et al. 1992, 1993, Dorgelo 1993, Sprung 1992, 1995a, Jantz 1996, Burlakova 1998, Jantz & Neumann 1998, Schneider et al. 1998, Horvath & Lamberti 1999
Water current	Moderate current accelerates growth	Kachanova 1963, Mikheev 1964, bij de Vaate 1991, Smit et al. 1992, 1993, Dorgelo 1993, Burlakova 1998
Depth	Growth decreases with depth	Mikheev 1964, Garton & Johnson 2000
Wave action	Inhibits growth rate	Mikheev 1964
Turbidity	High amount of suspended matter inhibits growth rate	Reeders et al. 1989, Noordhuis et al. 1992, Alexander et al. 1994, Summers et al. 1996, Madon et al. 1998, Schneider et al. 1998
Year-to-year variation	Growth varies significantly	Lvova 1980, Dorgelo 1993, Chase & Bailey 1999b

mussels form a distinct size class maintain distinct size structure for all age/size classes (Golikov 1970). However, in many water bodies *D. polymorpha* spawn throughout the entire summer, producing several peaks in veliger densities during the year (Lvova 1977, 1980, Karatayev 1983, Lvova et al. 1994, Burlakova 1998) and a wide size range (up to 16 mm difference in size) by the end of their first growing season (Wesenberg-Lund 1939, Mikheev 1964, Kirpichenko 1971, Szlauer 1974, Lvova 1977, Lewandowski 1983, Neumann et al. 1993, Martel 1995). Therefore, age classes will not form distinct size classes (Karatayev 1983, bij de Vaate 1991, Jantz & Neumann 1992, Lvova et al. 1994). This method is most effective when *D. polymorpha* spawn synchronously, have fast growth and are short lived, such as the shallow areas of the Svisloch River (Burlakova 1998). In this river *D. polymorpha* settle in the summer and grow during the following year, producing two distinct size classes of mussels (0+ and +) because the majority of older mussels die over winter because of fluctuating water levels and predation by ducks. At deeper depths mussels survive longer, producing many age classes, which are less distinct as cohorts, making this method less useful (Burlakova 1998). This method could also be used for studies that follow growth on experimental substrates when the time of settlement is known (Lvova 1977, Sprung 1992).

#### Growth Under Experimental Conditions

Many studies have been used to estimate *D. polymorpha* growth under experimental conditions, especially in cages. In the FSU this method was used in Kuybyshevskoe (Mikheev 1964), Uchinskoe (Lvova-Kachanova 1972, Lvova 1980) and Tsimlyanskoe (Lvova et al. 1983) reservoirs, in Lake Lukomskoe (Karatayev 1983), the Narochansk lakes and the Svisloch River (Burlakova 1998). More recently, this method has been used by East European (Stanczykowska & Lewandowski 1995), West Eu-

ropean (Smit et al. 1992, 1993, Sprung 1992, 1995a, Dorgelo 1993, Dall & Hamburger 1996) and North American scientists (Bitterman et al. 1994, MacIsaac 1994, Allen et al. 1999, Horvath & Lamberti 1999, Yu & Culver 1999, Garton & Johnson 2000).

Other methods for studying mussel growth under experimental conditions include growing mussels on artificial substrates (Dorgelo & Gorter 1984, Sprung 1992, Martel 1993), and in the laboratory (Walz 1978a, 1978b, Jantz & Neumann 1992, 1998, Dorgelo 1993, Neumann et al. 1993, Jantz 1996, Baldwin et al. 2002). All of these methods could produce different types of artifacts, which may influence observed growth rates. Mesh, usually 3–5 mm, may prevent normal water flow through the cage, particularly for smaller mesh sizes (e.g., 1.2 mm, Garton & Johnson 2000). Cages can also be overgrown by periphyton, further reducing water flow (Kachanova 1963, Karatayev 1983, Stanczykowska & Lewandowski 1995, Burlakova 1998). However, the effects of caging artifacts on growth rates are usually not well tested or quantified (see Burlakova 1998 later).

#### Marked Mussels Under Natural Conditions

Following tagged mussels under natural conditions has been used in very few studies (e.g., Stoeckman & Garton 1997, Burlakova 1998) although this method could provide the most realistic estimates of zebra mussel growth rates. Burlakova (1998) found that the growth rate of mussels on stones in the Svisloch River was greater than that for caged mussels in the same environment (Fig. 1A). Early in the spring (April), when macrophytes and periphyton abundances were low, the difference between caged and uncaged mussels was small (~30%). This difference increased to almost 400% in the middle and especially by the end of the growing season when the quantity of drifting plants in the water increased and periphyton densities were high (Burlakova 1998). The disadvantage of this method is that zebra mussels can move, form



TABLE 2.  
Estimates of the longevity of *Dreissena polymorpha* from different methods.

Waterbody	Longevity (years)	Maximum Length (mm)	Reference
Counts of annual rings on shells			
Volga River	18	32–33	Karpevich 1952
Volga River	17–19	30–32	Karpevich 1964
Uchinskoe Reservoir	11	32	Kachanova 1963
Pyalovskoe Reservoir	10–12	29–33	Mikheev 1964
Mazurian lakes	5–7, max 10	n.r.	Stanczykowska 1963
Firth Szczecin	5–6	30–35	Wiktor 1969
Volgogradskoe Reservoir	7–9	30	Spiridonov 1971
Masurian lakes	5	n.r.	Stanczykowska 1975
Koninskie lakes	4	29	Kornobis 1977
Lukomskoe Lake	6–8	32–34	Karatayev & Tishchikov 1979
Jorzec Lake	7	n.r.	Lewandowski 1982b
Glebokie Lake	5	n.r.	Lewandowski 1982b
Bartag Lake	5	n.r.	Lewandowski 1982b
Otow Lake	5	n.r.	Lewandowski 1982b
Majecz Wielki Lake	5	n.r.	Stanczykowska et al. 1983
Inulec Lake	5	n.r.	Stanczykowska et al. 1983
Zelwazek Lake	5	n.r.	Stanczykowska et al. 1983
Plas Leblance Pond	5	29.9	Draulans & Wouters 1988
Laguno Pond	4	25.7	Draulans & Wouters 1988
Tsimlyanskoe Reservoir	7	31–33	Miroshnichenko 1990
Average $\pm$ SE	7.4 $\pm$ 0.9		
Analysis of size-frequency distributions			
n.r.	3	31–34	Clarke 1952
Reservoir #2 Walthamstow	5	40	Morton 1969a
River Rhine	3	31	Jantz & Neumann 1992
Lake Esrom	4	32	Dall & Hamburger 1996
River Svisloch	3	30	Burlakova 1998
Lake Ontario:			
Stoney Point	2	n.r.	Chase & Bailey 1999a
Wheatley (2 and 6 m)	2–3	n.r.	Chase & Bailey 1999a
Port Dalhousie (2 and 6 m)	$\geq 4$	n.r.	Chase & Bailey 1999a
Average $\pm$ SE	3.3 $\pm$ 0.3		
Growth in experimental cages			
Uchinskoe Reservoir	4	36	Lyova 1980
Lukomskoe Lake	8	30	Karatayev 1984
Lake Wawasee	3	n.r.	Garton & Johnson 2000
Svisloch River	3	30	Burlakova 1998

n.r. = not reported

druses or be consumed by predators, making it difficult to follow individuals through time.

#### IMPACT OF ENVIRONMENTAL FACTORS ON GROWTH RATE

The growth rate of *Dreissena* depends on water temperature, season of the year, location in the water column, trophic conditions, which affect food availability, and water velocity as well as other environmental factors (Table 1).

##### Temperature

It is well established that the growth rate of *D. polymorpha* is accelerated by increased water temperature (Table 1). Especially convincing are data from studies in different temperature zones of cooling water reservoirs for thermal power plants in the FSU (Yaroshenko & Naberezhnyi 1971, Skalskaya 1976a, 1976b, Elagina et al. 1978, Karatayev & Tishchikov 1979, Karatayev 1983,

1984, 1988) and other areas of Eastern Europe (Stanczykowska 1976a, Kornobis 1977). In these studies *D. polymorpha* growth rates were compared among the various temperature zones of the same waterbody or lakes within same lake system. Therefore, environmental conditions other than temperature were similar, allowing a direct estimate of thermal effects.

However, when the maximum temperature is  $>30^{\circ}\text{C}$ , *D. polymorpha* growth decreases, and at temperatures  $>32^{\circ}\text{C}$  most mussels die. In the hottest zone of Lukomskoe Lake, where maximum summer temperature exceeds  $32^{\circ}\text{C}$ , more than 90% of the *D. polymorpha* in experimental cages died, whereas in the moderately heated zone (maximum summer temperature  $\leq 30^{\circ}\text{C}$ ) mortality was less than 10% and did not differ from the control, ambient zone (Karatayev 1983). Similar upper maximal temperature limits for *D. polymorpha* survival have been found by other authors in different regions of the FSU:  $31.5^{\circ}\text{C}$  in Zaporozhskoe Reservoir and  $32^{\circ}\text{C}$  in a canal of the Pridneprovskaya Power Station in

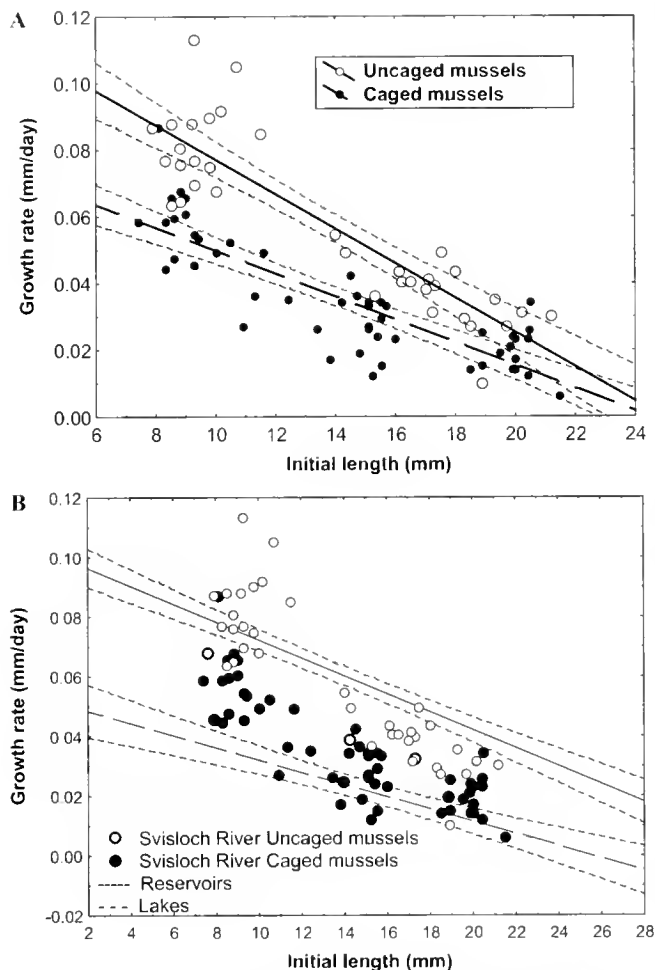


Figure 1. A. Growth rate (mm/day) of caged (filled circles) and uncaged (open circles) *Dreissena polymorpha* in the Svisloch River in 1995 (from Burlakova 1998). Data points are the growth rates of individual mussels. Regression lines (uncaged – solid line,  $Y = 0.129 - 0.005 \cdot X$ ,  $R^2 = 0.78$ ,  $P < 0.001$ ; caged – dashed line,  $Y = 0.084 - 0.003 \cdot X$ ,  $R^2 = 0.71$ ,  $P < 0.001$ ) and 95% confidence intervals are shown. Uncaged mussels grew faster than caged mussels. The two regression lines differ significantly in slope ( $P = 0.003$ ). B. Growth rate of caged and uncaged *D. polymorpha* in the Svisloch River with the regression lines for size-specific growth of *D. polymorpha* in cages in lakes (dashed line) and reservoirs (solid line). Caged mussels in the river grew at rates more similar to those caged in lakes, whereas uncaged mussels grew at rates more similar to those caged in reservoirs.

Ukraine (Lyakhovich et al. 1994), 32°C in Kuchurganskiy Liman in Moldova (Vladimirov 1983), 33°C in a cooling reservoir of the South-Ukrainian Nuclear Power Station (Sinitsina & Protasov 1993) and 34°C in a cooling reservoir of the Chernobyl Nuclear Power Station (Protasov et al. 1983).

The upper temperature limit for *D. polymorpha* in European cooling reservoirs is similar to that found in the Lower Mississippi River, where maximum temperature in the summer exceeds 30°C (Allen et al. 1999). In this river *D. polymorpha* growth rate is highest during spring and fall when temperatures ranged from 16°C to 28°C, however, shell growth ceases during the summer when temperatures remain 29°C to 31°C (Allen et al. 1999). Mihuc et al. (1999) found that in the Atchafalaya River system (Louisiana) *D. polymorpha* grow throughout the winter and growth in-

creases in late spring (April and May). Adult mortality occurs from May to August because dissolved oxygen levels decline and minimum daily temperatures warm above 29°C in the floodplain and 32.5°C in riverine sites. Thus, the maximum upper temperature limit for *D. polymorpha* survival is similar in both Europe and North America.

#### Season

In temperate regions, zebra mussel growth stops in the winter and resumes in the spring after water temperatures warm. Although Smit et al. (1992) assumed that the lower temperature limit for shell growth should be set by the lower temperature limit for filtering (3°C; Mikheev 1967a, 1967b, Kondratiev 1969, Reeders & bij de Vaate 1990), a majority of studies have found that the threshold temperature for mussel growth is 10°C to 12°C (12°C, Kachanova 1961; 11°C, Morton 1969a, 1969b; 10°C, Alimov 1974, Karatayev 1983, Jantz & Neumann 1992; 10°C to 12°C, Mackie 1991). bij de Vaate (1991) did report a lower temperature limit of 6°C, however in North America MacIsaac (1994) found that small mussels incubated at 6°C experienced shell degrowth and mass loss, whereas large individuals experienced shell degrowth but weight gain. These differences among studies may be the result of local effects, but clearly call for further study.

Maximum growth in *D. polymorpha* is usually found early in the growing season (Karatayev 1983, 1984, Smit et al. 1992, Lvova et al. 1994, Burlakova 1998, Garton & Johnson 2000), and corresponds with a peak in phytoplankton abundance (Walz 1978a). In midsummer growth rate often decreases (Spiridonov 1971, Walz 1978a, Smit et al. 1992, Stanczykowska & Lewandowski 1995, Allen et al. 1999) and has been attributed to low food concentrations (Walz 1978a), blooms of dinoflagellates (e.g., *Ceratium hirundinella*) that impede filter feeding (Stanczykowska & Lewandowski 1995), high water temperatures (Allen et al. 1999) and spawning (Spiridonov 1971, Lvova 1977, 1980, Karatayev 1983, 1992, Allen et al. 1999). In the autumn, when water temperatures decrease, growth stops (Morton 1969a). In the Uchinskoe Reservoir growth stops when temperatures fall to 10°C (Lvova 1977, 1980) and in the River Rhine at 10° to 15°C (Jantz & Neumann 1992).

Based on a bioenergetics model of zebra mussel growth in the Laurentian Great Lakes, Schneider (1992) predicted positive growth in the spring and fall when high phytoplankton biomass associated with spring and fall turnover coincides with temperatures near the optimum for growth. Even under conditions of high food availability, growth rates in his model typically decline in the beginning of summer and increase again in August and September as temperature begins to decline. Schneider (1992) used Walz's bioenergetic estimates of metabolic parameters for zebra mussels from Lake Constance (Walz 1978d), where the optimal temperature range for growth is 8°C to 15°C. This temperature range is much lower than optimum found by other authors, and therefore more empirical tests of this model are needed before we can assess the generalizability of its predictions.

#### Location in the Water Column

*Dreissena polymorpha* grow faster in the water column above the bottom (e.g., on buoys, cages, submerged constructions, floating objects) than on the bottom (Kachanova 1963, Mikheev 1964, bij de Vaate 1991, Smit et al. 1992, 1993, Dorgelo 1993, Burlakova 1998). Yu and Culver (1999) tested the effect of cage loca-

tion in stratified Hargus Lake (Ohio), and found highest growth at their pelagic site (2.5–4 m depth) and in the littoral zone at 2.5 m depth. All mussels held below the thermocline (5-m depth) died before the end of experiment (163 days).

#### Trophic Conditions

Trophic conditions also affect zebra mussel growth (Table 1). Dorgelo (1993) found that zebra mussels growth rates in Dutch eutrophic lakes Mararsveen II and Vechten was higher ( $0.54\text{--}0.59\text{ mm wk}^{-1}$ ) than of those grown in mesooligotrophic Lake Maarsveen I ( $0.35\text{ mm wk}^{-1}$ ), even though there was no difference in mean temperature between these lakes. Jantz and Neumann (1992) found a significant strong correlation ( $r_s = 0.80$ ) between the rate of shell length growth and chlorophyll *a* concentration and between shell growth and temperature ( $r_s = 0.82$ ). However, because these two environmental factors are highly correlated, it is impossible to determine the relative contribution of each of these factors on growth (Jantz & Neumann 1992). In a later study (Jantz & Neumann 1998) they found that shell growth rate and the duration of the growing season were correlated with the quantity of available algal food.

Sprung (1995a) found a strong correlation between zebra mussel shell growth and food conditions (siston concentration). He suggested that this correlation will exist when seston concentrations stay below those at which the intestine is filled to capacity when the animal filters at a maximum rate (Sprung 1995b). Similarly, Schneider et al. (1998) found that the scope for growth under laboratory conditions had a strong positive relationship with food quality.

#### Water Motion

In areas with constant water current *D. polymorpha* grow faster than in still water (Table 1). Kachanova (1963) found that *D. polymorpha* grow faster on the concrete walls of the canal flowing from Uchinskoe Reservoir than in the reservoir. Mikheev (1964) found that in Kuybyshevskoe Reservoir moderate water currents (up to  $0.8\text{ m s}^{-1}$ ) facilitate mussel feeding and respiration and *D. polymorpha* grown in water currents reached 27–28 mm, whereas same aged mussels at the same depth out of currents were only 19–20 mm in length. Smit et al. (1993) suggested that water movement seems to have a larger influence on growth than the amount of algal food in the water column. They found that the young-of-the-year zebra mussels in the Rhine River were almost 3 times longer (16 mm) than in Lake IJsselmeer (6 mm) in spite of lower chlorophyll *a* concentrations in the river ( $10\text{--}42\text{ }\mu\text{g L}^{-1}$  in the river,  $34\text{--}106\text{ }\mu\text{g L}^{-1}$  in lakes).

However, strong water currents may inhibit *Dreissena* growth. The maximum length of 2-y-old zebra mussels in waterways of the Kuybyshevskaya hydroelectric power plant with constant water currents  $<0.5\text{ m s}^{-1}$  was 18 mm, and in places with water currents  $>1.5\text{ m s}^{-1}$  was 13–14 mm (Mikheev 1964).

Wave action can also inhibit *D. polymorpha* growth. Mikheev (1964) found that in the littoral zone of Kuybyshevskoe Reservoir exposed to waves, the average (4–5 mm) and maximum (8–10 mm) length of yearling mussels was almost half that of mussels at the same depth but without waves (7.2 mm mean, 14 mm maximum). He also found that the average length of the young-of-the-year *D. polymorpha* in parts of the Tsimlyanskoe Reservoir exposed to strong waves was 9 mm (maximum 12.5 mm), whereas in

quiet areas at the same depth the average length was 12 mm (maximum 19.2 mm).

#### Depth

*Dreissena polymorpha* grow faster in shallow than in the deep parts of a waterbody (Table 1). In Kuybyshevskoe Reservoir the maximum length of yearling mussels at 1–1.5 m depth was 13.7–14 mm, and at 20 m depth, only 6–7 mm (Mikheev 1964). Similarly, Garton and Johnson (2000) found that in Lake Wawasee zebra mussel growth rate declined 15% per meter between 1–4 m depth. They hypothesized that this decrease was caused by lower temperature and reduced food with depth.

#### Turbidity

High concentrations of suspended matter in the water negatively affects filtration, ingestion, assimilation and growth potential of zebra mussels (Reeders et al. 1989, Noordhuis et al. 1992, Alexander et al. 1994, Summers et al. 1996, Madon et al. 1998, Schneider et al. 1998). In Dutch lakes, clearance rates of adult 20-mm zebra mussels declined exponentially as dry suspended matter increased from  $5\text{--}90\text{ mg L}^{-1}$  (Reeders et al. 1989, Noordhuis et al. 1992).

Madon et al. (1998) found that concentrations of suspended inorganic sediment above  $1\text{ mg L}^{-1}$ , and a ratio of inorganic to organic fraction of seston higher than 1.71 may cause negative growth. Similar limits were found by Schneider et al. (1998): the scope for growth declined with decreasing food quality and fell below  $0\text{ cal mg}^{-1}\text{ h}^{-1}$  at an organic/inorganic ratio of 0.5. They suggested that high concentrations of suspended inorganic sediment in large turbid rivers represents a difficult growth environment for zebra mussels and that populations in turbid rivers may not stabilize at the very high densities typical of lentic environments.

#### Year-to-year Variation

The growth rates of *D. polymorpha* in the same waterbody vary significant among years (Lvova 1980, Dorgelo 1993, Chase & Bailey 1999b). Zebra mussels in the Uchinskoe Reservoir with initial shell lengths of 8 mm grew to  $21.2 \pm 0.29\text{ mm}$  by the end of the growing season in 1967, to  $19.5 \pm 0.27\text{ mm}$  in 1968, and to  $16.8 \pm 0.18\text{ mm}$  in 1969 (Lvova 1980). Dorgelo (1993) found the growth rate of *D. polymorpha* in lakes Maarsveen I and II was significantly lower in the summer of 1986 than in 1985. In a study of growth and production of *D. polymorpha* in lakes St. Clair, Erie and Ontario, Chase and Bailey (1999b) estimated shell production as a part of total production (total production = shell + somatic + gamete production). They found that the site by year interaction (among 5 populations) explained  $>80\%$  of the variation; differences between sites in production depended on the year examined. Variation in total production depended on variation in somatic and shell production only, as gamete production was relatively constant among years. Chase and Bailey (1999b) hypothesized that, in response to poor environmental conditions, *D. polymorpha* shifts the allocation of resources from growth (somatic and shell) to reproduction. As individuals cannot predict how long adverse conditions will persist, investment in growth may be unprofitable (Chase & Bailey 1999b).

#### Lakes versus Reservoirs

Although environmental factors that affect mussel growth such as temperature, food availability and other conditions can differ

greatly among waterbodies and among years within the same waterbody, when we compared size-specific growth rates of zebra mussels among studies that all used a similar method (following caged mussels), some patterns emerged. We compared the data for size specific zebra mussel growth from three studies in reservoirs (two different reservoirs, two different years in one reservoir) and six studies conducted in 5 different lakes (4 in Eastern Europe, 1 in North America). We found that the size-specific growth of mussels in reservoirs was consistently higher than that of mussels grown in lakes (Fig. 2). Surprisingly, given the range of likely conditions among water bodies and among years, growth in reservoirs was very consistent ( $R^2 = 0.92$ ). For lakes there was more spread ( $R^2 = 0.72$ ), but the patterns and rates were similar among studies. Data for the growth of uncaged mussels in the Svisloch River are much more similar to mussels in reservoirs, whereas those grown in cages were more similar to mussels in lakes (Fig. 1 B).

### LIFE SPAN

#### Methods to Estimate Longevity

Most of the methods used to estimate longevity of *D. polymorpha* are similar to those used to estimate growth rate: counting annual rings on shells, analysis of the size-frequency distributions and growth under experimental conditions (Table 2).

#### Counting Annual Rings on Shell

The maximum longevity reported using this method has decreased through time from 17–19 y (Karpevich 1964) to 4–5 y

(Draulans & Wouters 1988). Through time some authors have revised their earlier estimates of zebra mussel longevity. Stanczykowska (1964) initially reported a maximum longevity for *D. polymorpha* of 10–12 y; 11 y later she revised her estimates from these same data to 5 y (Stanczykowska 1975, 1976b). Kachanova (1963) (this author published later under the name Lvova-Kachanova and Lvova) reported that the maximum life span of zebra mussels in the Uchinskoe Reservoir was 6–11 y, and later revised this estimate to 4 y (Lvova 1980, Table 2). Although the advantage of this method is that it allows an estimate of the age structure of a population by measuring *D. polymorpha* at a single point in time, as discussed earlier, it is very difficult to separate annual rings formed during winter from other rings.

#### Analysis of Size-Frequency Distributions

For this method the numbers of peaks on a size-frequency histogram are counted, assuming that each peak represents an age class. However, as discussed earlier, age classes may not have distinct sizes, making it difficult to estimate longevity based on size-frequency distributions.

#### Growth Under Experimental Conditions

Usually authors keep mussels of different initial sizes in cages for a limited period (1–4 y), and then the obtained growth rates are used to estimate the time to reach the maximum size found in the population. However keeping mussels in cages can produce different types of artifacts discussed earlier, which may affect observed growth rates and, therefore, estimates of mussel longevity.

#### Factors Affecting Longevity

To our knowledge, the first estimates of the longevity of zebra mussels were reported by Karpevich (1952) and Clarke (1952) (Table 2). Karpevich (1952) counted annual rings on shells and estimated zebra mussel longevity in the Volga River as 18 y. In contrast, Clarke (1952) using unpublished data from J. Wilhelm (study site not mentioned) found three peaks in the size-frequency distribution of *D. polymorpha* and suggested that typical longevity is about three years. Overall, the longevity of *D. polymorpha* estimated by different authors over the last 50 y varies from 2–19 y. However, the maximum sizes of *D. polymorpha* reported by these authors are similar (Table 2). This contradiction supports a hypothesis that the reported differences in longevity may be explained to a large extent by the artifacts of the methods used. This suggestion is also supported by the fact that the average *D. polymorpha* longevity estimated by counting annual rings on shells ( $7.4 \pm 0.9$ ) is significantly different from average longevity estimated by analysis of size-frequency distribution ( $3.3 \pm 0.3$ ,  $P < 0.001$ , 2-sided *t*-test) (Table 2). Alternatively, zebra mussels may have a fixed maximum size, and local conditions that affect growth rates determine longevity—fast growing mussels will live for shorter periods of time, whereas slow growing mussels will live longer. Therefore, it is unclear how much of this variability in longevity is natural or is caused by the artifacts of the methods used and definitely requires future investigation.

### GENERAL FINDINGS AND FUTURE DIRECTIONS

Although many generalizations can be made about the growth rate and longevity of *D. polymorpha*, and the impacts of various environmental factors on these parameters, the answers to many questions are far from clear. The most important questions that

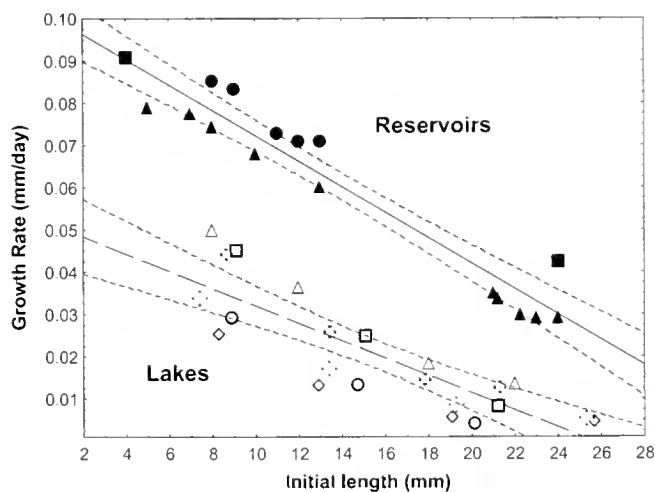


Figure 2. Growth rates of *Dreissena polymorpha* in reservoirs (filled symbols) and lakes (open symbols, stars and crosses). In all studies, zebra mussels were kept in cages and followed through time. Regression lines are for size-specific growth rate in reservoirs (solid line,  $Y = 0.102 - 0.003 \cdot X$ ,  $R^2 = 0.92$ ,  $P < 0.001$ ) and lakes (dashed line,  $Y = 0.053 - 0.002 \cdot X$ ,  $R^2 = 0.72$ ,  $P < 0.001$ ) with 95% confidence intervals. The two regression lines significantly differ in slope ( $P = 0.013$ ) and their 95% confidence intervals do not overlap. Data sources are: ● Uchinskoe Reservoir, 1967 and ▲ Uchinskoe Reservoir, 1968 (Russia)—Lvova (1980), ■ Tsimlyanskoe Reservoir (Russia)—Mironshchenko (1990), ○ Lake Lukomskoe, unheated zone and □ Lake Lukomskoe, heated zone (Belarus)—Karatayev (1983), △ Mikolajskie Lake (Poland)—Stanczykowska & Lewandowski (1995), ◇ Lake Naroch (Belarus)—Burlakova (1998) and + Lake Myastro (Belarus)—Burlakova (1998), \* Lake Wawasee (US)—Garton & Johnson (2000).

need to be addressed, problems that need to be solved, and targets for future study are:

#### Methodological Problems

Growth rate and longevity of *D. polymorpha* have been estimated by using four different methods, most of which have serious methodological problems. Thus, different estimates of *D. polymorpha* growth rates and longevity are affected not only by differences in environmental conditions but also by artifacts of the methods used. Following the growth of undisturbed *D. polymorpha* will provide more reliable data on growth potential and variability among different waterbodies with different environmental conditions. Following and subsampling mussels that naturally settle on experimental surfaces through time provides the control and ease found in experimental studies with the growth rates expected from natural populations.

#### Co-effects of Environmental Factors

*Dreissena polymorpha* growth rates depend on water temperature, season of the year, location in the water column, food availability, oxygen concentrations, water velocity and various other environmental factors (Table 1). However, it is difficult to separate the independent effects of each of these factors, especially in natural waterbodies where most of these factors will covary. Several factors may have additive or synergistic effects, making it difficult to study the effect of a single factor. Separation of the effects of single and combined factors on growth is essential.

#### Temperature

The upper temperature limit for zebra mussel growth seems to be 30°C to 32°C, and the lower temperature limit ~10°C to 12°C (Kachanova 1961, Morton 1969a, 1969b, Alimov 1974, Karatayev, 1983, Mackie 1991, Jantz & Neumann 1992). However, some studies have found much lower limits (bij de Vaate

1991, Smit et al. 1992). Differences among studies may be a result of local effects but clearly calls for further study.

#### Growth in Different Types of Waterbodies

There seems to be substantial differences in growth between mussels in reservoirs and lakes—mussels grow much faster in reservoirs than lakes. Experiments that directly test the relative contributions of environmental factors versus the type of water body and what factors are different between reservoirs and lakes are clearly called for to answer this question. It may be that reservoirs provide a better overall growth environment in terms of temperature, nutrition, and water motion than do natural lakes or rivers.

#### Longevity

The reported longevity of *D. polymorpha* varies from 2 to 19 y. It is critically important to understand to what extent this variation is caused by biological variability, environmental conditions and what amount of the variation is caused by the methods used. In addition to the basic value of understanding the variability in *D. polymorpha* longevity, it is also important if we are to predict population dynamics, spread or to develop control methods for this important invader.

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## POPULATION STRUCTURE IN TWO MARINE INVERTEBRATE SPECIES (*PANOPEA ABRUPTA* AND *STRONGYLOCENTROTUS FRANCISCANUS*) TARGETED FOR AQUACULTURE AND ENHANCEMENT IN BRITISH COLUMBIA

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**ABSTRACT** As the number of native species targeted for aquaculture and enhancement in British Columbia (BC) rises, fisheries managers must develop policies and species-specific management plans that facilitate industry expansion while protecting the integrity and fitness of wild stocks. Whereas genetic introgression of domesticated stocks with wild stocks cannot be completely eliminated, some control can be gained by limiting the collection and culture of domesticated stocks to geographic units defined by the genetic structure of wild stocks. We describe the genetic structure of two species that are targeted for intensive enhancement and aquaculture in BC: Geoduck clams, *Panopea abrupta* and red sea urchins, *Strongylocentrotus franciscanus*. Based on a survey of eight polymorphic microsatellite loci, *P. abrupta* contained significant geographic structure within BC, with the distribution of genetic variation consistent with stepping stone gene flow under an isolation-by-distance model. A survey of seven polymorphic microsatellite loci covering a similar geographic range revealed genetic homogeneity of *S. franciscanus* in BC. Based on the different levels of structure displayed in the two species, we propose three geographically-based management units for *P. abrupta* and two for *S. franciscanus* in BC.

**KEY WORDS:** geoduck, clam, red sea urchin, *Panopea abrupta*, *Strongylocentrotus franciscanus*, population structure, enhancement, genetic, microsatellite

### INTRODUCTION

In British Columbia (BC), many native (mussels) and nonnative (oysters, manila clams, Japanese scallops, Atlantic salmon) marine species are under intensive aquaculture. Additional species that are important capture fisheries are also becoming prospects for aquaculture and artificial enhancement (e.g., geoduck, urchins, blackcod, halibut and rockfish species). Different genetic issues accompany the widespread hatchery production of native and nonnative species. In the culture of nonnative species, competition and hybridization with native species are potential impacts, whereas in the culture of native species, reduction of the genetic fitness of wild populations through introgression with aquaculture strains is a primary concern (Utter & Epifanio 2002). There are three ways in which hatchery-reared animals may differ genetically from their wild counterparts. They may have a geographically distant origin and may be adapted to different natural conditions. Such organisms released from the hatchery may be relatively unfit in their new environment and hybridization with wild conspecifics may lead to outbreeding depression, a reduction in fitness of hybrids in the wild (Allendorf & Ryman 1987). When local organisms are used for hatchery propagation, the reduced number of parents successfully contributing to hatchery offspring can lead to founder effects, genetic drift and future inbreeding (Tave 1993). Even when large numbers of broodstock are collected locally, domestication, or adaptation to hatchery culture, may reduce the fitness of hatchery-produced organisms and their hybrids in the wild (Lynch & O'Hely 2001, Ford 2002).

Few species have been subjected to the detailed studies required to detect and quantify fitness differences between wild, cultured and hybrid organisms in the wild environment. For Atlantic salmon, one of the most intensively cultured and studied species worldwide, domesticated fish and their first-generation hybrids with wild fish have lower fitness than wild fish in natural

environments (McGinnity et al. 1997, Fleming et al. 2000, McGinnity et al. 2003). For invertebrate species, hatchery-produced organisms often contain reduced genetic variability compared with their wild conspecifics (Hedgecock et al. 1992, Boudry et al. 2002), but few studies have investigated the fitness of cultured individuals in different environments (see Tremblay et al. 2001, Jorstad et al. 2005, Sekino et al. 2005).

Because most cultured marine invertebrate species have pelagic larval dispersal stages, complete isolation of wild and hatchery-reared conspecifics could only be achieved by limiting culture to expensive on-land facilities, rearing of nonreproductive animals (e.g., triploids) or complete harvesting of cultured animals before they become reproductively mature. However, introgression of geographically distinct cultured stocks can be avoided by managing broodstock collection and outplanting within regions identified on the basis of wild population structure. Loss of variation and domestication in hatchery strains can be reduced by maintaining large broodstock numbers of wild origin organisms for hatchery production. Alternately, in the case where hatchery broodstocks are genetically distinct from wild stocks, either having undergone extensive genetic selection, bottlenecks or genetic marking, prior knowledge of the genetic characteristics of the wild populations and local hatchery broodstocks may enable monitoring in the vicinity of aquaculture sites and beyond to detect genetic interactions.

Most marine invertebrate species currently cultured or enhanced are characterized by high levels of fecundity and planktotrophic larvae with high dispersal capabilities, both features that are associated with genetic panmixia (Bohonak 1999). However, although panmixia is often observed in marine invertebrate species with these life history attributes, many species do not display panmixia over all spatial scales (Hellberg et al. 2002). The three additional types of structure most often observed include chaotic genetic patchiness, isolation by distance (IBD) and abrupt phylogenetic breaks associated with geographic barriers (reviewed in Hellberg et al. 2002). Chaotic genetic patchiness, defined by non-

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geographically based genetic variance over space and/or time, has been observed over small to medium geographic scales (0–500 km) in a large range of marine invertebrate species (Johnson & Black 1982, Benzie & Williams 1997, Michinina & Rebordinos 1997, Moberg & Burton 2000, Hamm & Burton 2000). An IBD model of structure results from stepping stone gene flow (Slatkin 1993), and has been observed over macrogeographic (100s to 1,000s of km) scales in some marine invertebrate species (Shaklee & Bentzen 1998, Hellberg et al. 2002, Palumbi 2002). Alternately, abrupt genetic boundaries have been observed most often in species occupying bays and estuaries, or those crossing strong phylogeographic boundaries limited by ocean current circulation (Reeb & Avise 1990, Hellberg et al. 2002).

Geoduck clams, *Panopea abrupta* (hereafter *Pab*) and red sea urchins, *Strongylocentrotus franciscanus* (hereafter *Sfr*) are both long lived (up to 100–150 y) mainly dioecious species that form dense aggregations in the wild (Bureau et al. 2002, Ebert & Southon 2003). Larval duration (approximately 6 wk) is similar in both species, and repeat broadcast spawning events synchronized among individuals clustered over small spatial scales occur from spring through summer (Ebert et al. 1994, Goodwin & Bradbury 2001, Flowers et al. 2002). Because recruitment is sporadic, natural replenishment of over-fished sites is slow, with an estimated 30–50 y natural recovery time to preharvest levels for geoduck clams (Goodwin & Bradbury 2001). Enhancement with hatchery-reared juveniles to facilitate rebuilding has been in effect for intertidal geoduck clams in Washington State since 1982 and for

subtidal geoduck clams in BC (limited to the Strait of Georgia) since 1993 ([http://www-sci.pac.dfo-mpo.gc.ca/geoduck/intro\\_e.htm](http://www-sci.pac.dfo-mpo.gc.ca/geoduck/intro_e.htm)). Recent experimental work on outplanting methods on red sea urchins has also been undertaken (Alan Campbell, pers. comm.).

Little is known about the genetic structure of *Pab* and *Sfr* in BC, although some genetic information exists on these species from other geographic locations, and genetic data is available on species closely related to *Sfr*. In this study we examine the genetic structure of geoduck clams and red sea urchins in BC with highly variable microsatellite loci (Kaukinen et al. 2004, Miller et al. 2004) and use the information to provide genetic guidelines for culture activities.

## METHODS

*Pab* siphon tissue was collected from 16 sites ranging from Washington through northern BC and spanning over 1100 km of coastline (Fig. 1). Collections took place over a 3-y period, from 2000–2003. Ethanol preserved siphon tissue was extracted using the chelex method from Small et al. (1998). Variation at 2 dinucleotide (*Pab* 5, *Pab* 132) and 6 tetranucleotide (*Pab* 6, *Pab* 117, *Pab* 101e, *Pab* 105e, *Pab* 106e, *Pab* 112e) microsatellite loci isolated from *Pab* was surveyed using the primers and protocols outlined in Kaukinen et al. 2004 (note *Pab* 5 and 6 are from Vadopalas & Bentzen 2000).

*Sfr* were collected over 3 years from 13 sites spanning over

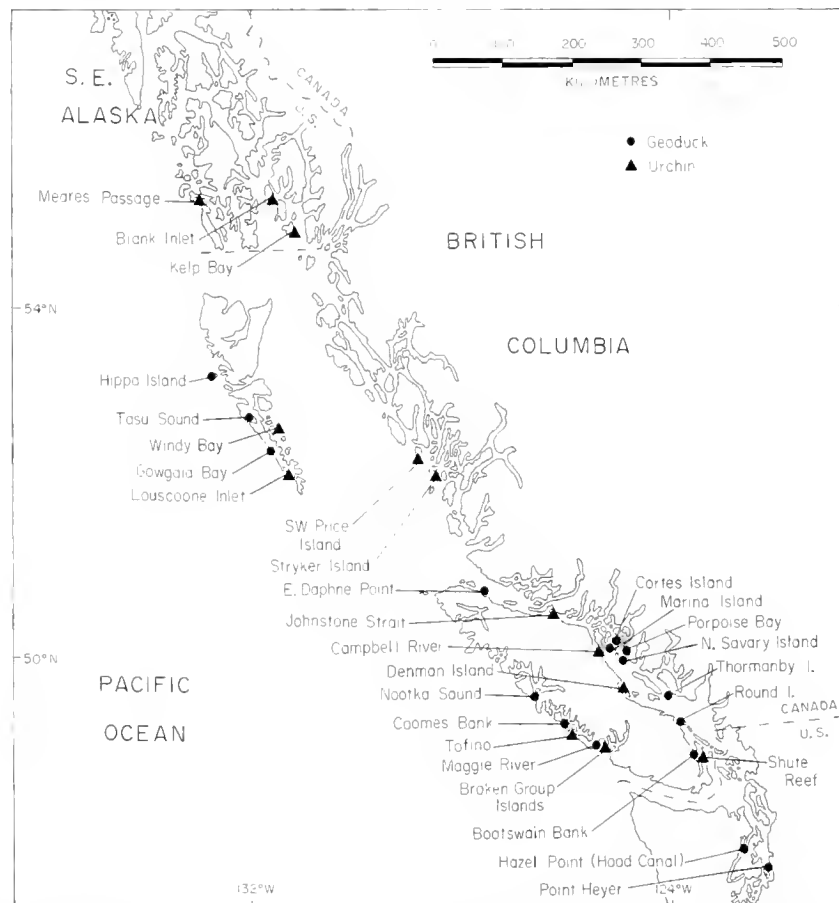


Figure 1. Map showing locations of *P. abrupta* and *S. franciscanus* collection sites in Washington, BC and Alaska. Collections were made for each species spanning a 3-y period, from 2000–2003.

1,000 km of coastline ranging from southern BC to Southern Alaska (Fig. 1 and Table 1). For *Sfr*, gonad tissue was collected destructively and frozen at  $-80^{\circ}\text{C}$  until use. Genomic DNA was extracted using DNeasy kits (Qiagen, Valencia, CA). Variation at six polymorphic dinucleotide (*Sfr* 06, *Sfr* 13, *Sfr* 22, *Sfr* 34, *Sfr* 64 and *Sfr* 90) loci and one trinucleotide (*Sfr* 55) repeat microsatellite locus isolated from *Sfr* was surveyed using the primers and protocols outlined in Miller et al. (2004).

Allelic and genotypic frequency data were analyzed using the Genetic Data Analysis (GDA) (Lewis & Zaykin 2001), GENEPOP version 3.1d (Raymond & Rousset 1995) and FSTAT version 2.9.3.2 (Goudet 2001). Conformance to Hardy Weinberg equilibrium (HWE) distributions of genotypic frequencies at each locus in each sample was tested using GENEPOP. Levels of differentiation among populations using  $F_{ST}$  values (Weir & Cockerham 1984) were computed over all samples and on a pairwise basis between samples using FSTAT. For loci that were not in HWE,  $F_{ST}$  distances were calculated by randomized genotypes rather than alleles (Goudet 2001). The significance of the multilocus  $F_{ST}$  value over all samples was determined by jackknifing over loci. Allelic richness was calculated by standardizing allelic diversity to a sample size of 50 individuals in FSTAT. Homogeneity of allele richness and heterozygosity among sites was tested using a chi-square ( $\chi^2$ ) test. An IBD model of structure was tested by regression of pairwise  $F_{ST}$  values with geographic distance (shortest waterway distances between sites), calculated in FSTAT following Mantel (1967). The stability of the IBD model structure was tested using the web-based IBD program from <http://www.bio.sdsu.edu/pub/andy/BohonakCV.html#Computer%Programs>, with all values set to default (Bohonak 2002).

Genetic relationships among samples of *Sfr* and of *Pab* were determined using a dendrogram based on the neighbor-joining algorithm, constructed using pairwise differences (Nei 1978). The effective population size ( $N_E$ ), which is an estimate of the number of breeding adults in a population, for *Sfr* and *Pab* was calculated from expected heterozygosity ( $H_E$ ) values over all loci using the relationship  $N_E = (1/[1-H_E]^2 - 1)/8\mu$ , where  $\mu$  is the mutation rate for the microsatellite loci (Lehmann et al. 1998). The mammalian mutation rate of  $\mu = 10^{-4}$  was used as a conservative estimate, recognizing that  $N_E$  values could be 100 times greater if  $\mu$  approaches  $10^{-6}$ , as observed in *Drosophila* (Shug et al. 1997).

Hierarchical analysis of allele frequency variation was conducted in Arlequin with a nested analysis of molecular variance (AMOVA; random effects model; Weir 1996). The significance of allele frequency differences attributable to geographic regions identified in the dendrograms based on  $F_{ST}$  distances was tested in a hierarchical model with sample sites nested within regions.

## RESULTS

### Genetic Variation Within Samples

*Pab* microsatellite loci were all highly polymorphic. Allelic richness ranged from 16–38 alleles per 50 individuals, and expected heterozygosities ranged from 0.91–0.98 among loci (Table 2). Four of the eight *Pab* loci contained excess homozygotes resulting in significant deviations from HWE over all sites, whereas *Pab* 112e contained an excess at 11/16 sites (overall  $P < 0.001$  for each of these loci). Deviations from HWE were minimal in the remaining three loci ( $P > 0.1$  over all sites). Estimates of the inbreeding coefficient,  $F_{IS}$ , over all sites ranged from 0.03 for *Pab* 6 to 0.30 for *Pab* 117 (Table 2).

TABLE 1.

Distribution of collection sites for *P. abrupta* and *S. franciscanus* in BC, Washington (WA), and Alaska (AK), with site-specific data on sample size, allelic richness ( $A_R$ ), and expected heterozygosity ( $H_E$ ).

Collection Site	Province or State	Region	Sample Size	$A_R$	$H_E$
<i>Pab</i>					
Point Heyer	WA	S. Puget Sound	98	28.0	0.967
Hazel Point	WA	Hood Canal	134	27.0	0.970
Marina Island	BC	ECVI	122	28.6	0.970
Savory Island	BC	ECVI	95	28.7	0.967
Cortes Island	BC	ECVI	92	27.7	0.961
Porpoise Bay	BC	ECVI	98	27.5	0.962
Round Island	BC	ECVI	99	28.1	0.970
Boatswain Bank	BC	ECVI	97	26.3	0.958
Thormanby Island	BC	ECVI	97	28.2	0.971
East Daphne Pt	BC	N-ECVI	98	26.3	0.962
Nootka Sound	BC	WCVI	103	26.7	0.967
Coomes Banks	BC	WCVI	102	27.8	0.969
Maggie River	BC	WCVI	96	26.4	0.966
Hippa Island	BC	QCI	137	27.5	0.970
Tasu Sound	BC	QCI	103	27.4	0.969
Gowgata Bay	BC	QCI	107	26.8	0.963
Mean			105.3	27.4	0.966
<i>Sfr</i>					
Shute Reef	BC	SCVI	84	19.8	0.933
Denman	BC	ECVI	68	20.3	0.943
Campbell River	BC	ECVI	78	19.2	0.937
Johnstone Strait	BC	N-ECVI	92	20.0	0.942
Broken Group	BC	WCVI	58	19.7	0.919
Tofino	BC	WCVI	79	19.4	0.943
Stryker	BC	CC	67	19.2	0.946
SW Price	BC	CC	63	19.7	0.944
Louscoone	BC	QCI	87	20.4	0.938
Windy Bay	BC	QCI	76	21.2	0.948
Kelp Bay	AK	SEAK	81	21.0	0.947
Blank	AK	SEAK	77	20.3	0.941
Meares Pass	AK	SEAK	72	19.4	0.944
Mean			75.5	20.0	0.941

Abbreviations include: Washington (WA), BC (BC), Alaska (AK), East coast of Vancouver Island (ECVI), north ECVI (N-ECVI), west coast of Vancouver Island (WCVI), Queen Charlotte Islands (QCI), central coast (CC), and south east Alaska (SEAK). Allele frequencies for all samples surveyed in this study are available at: [http://www.pac.dfo-mpo.gc.ca/sci/aqua/bgsid\\_e.htm](http://www.pac.dfo-mpo.gc.ca/sci/aqua/bgsid_e.htm).

*Sfr* microsatellite loci were slightly less polymorphic than *Pab* loci, with a range in allelic richness of 9–35 (mean 20) and a range in expected heterozygosity of 0.63–0.96. Fewer deviations from HWE were observed in *Sfr* than for *Pab*, with only two of the seven loci containing significant excesses of homozygotes in over 50% of the *Sfr* sites. As a result, the mean  $F_{IS}$  over all loci was lower in *Sfr* ( $0.11 \pm 0.03$ ) than in *Pab* ( $0.15 \pm 0.04$ ).

Using the mammalian microsatellite mutation rate ( $10^{-4}$ ) and  $H_E$  values estimated from the loci in this study, locus-specific estimates of effective population sizes ( $N_E$ ) for *Pab* ranged from 1.4–13.8 million (mean of 5.8 million), and for *Sfr* ranged from 0.08–9.7 million (mean of 2.1 million).

### Genetic Variation Among Samples

In *Pab*, levels of heterozygosity were homogeneously distributed among sites ( $P > 0.05$ ), whereas spatial heterogeneity in al-

TABLE 2.

Statistics on *S. franciscanus* and *P. abrupta* microsatellite loci, including allelic richness ( $A_R$ ), expected ( $H_e$ ), and observed ( $H_o$ ) heterozygosity, inbreeding coefficient ( $F_{IS}$ ), number of populations that deviate significantly ( $P < 0.05$ ) from HWE after bonferroni correction, Coancestry coefficient ( $F_{ST}$ ), and the associated  $P$ -value for  $F_{ST}$ . HWE was not assumed in the calculation of the Coancestry coefficient ( $F_{ST}$ ) when 20% or more of the populations significantly deviated from HWE (highlighted with an asterisk).

Locus	N	$A_R$	$H_e$	$H_o$	$F_{IS}$	# Pop $P < 0.05$	$F_{ST}$ HW	GP-value
<i>P. abrupta</i>								
<i>Pab</i> 5 <sup>1</sup>	1118	16	0.91	0.71	0.22	16	0.0040*	0.002
<i>Pab</i> 6 <sup>1</sup>	1113	22	0.93	0.90	0.03	0	0.0032	0.002
<i>Pab</i> 117	1019	35	0.97	0.68	0.30	16	0.0020*	0.002
<i>Pab</i> 132	1019	38	0.94	0.70	0.26	15	0.0040*	0.002
<i>Pab</i> 101e	1113	19	0.93	0.88	0.05	3	0.0024	0.002
<i>Pab</i> 105e	1113	38	0.98	0.72	0.22	16	0.0020*	0.002
<i>Pab</i> 106e	1018	18	0.93	0.91	0.06	5	0.0041*	0.002
<i>Pab</i> 112e	1113	34	0.96	0.88	0.09	11	0.0050*	0.002
Mean		27	0.94	0.80	0.15		0.0036	
<i>S. franciscanus</i>								
<i>Sfr</i> 06	1016	12	0.87	0.85	0.03	0	0.0000	0.468
<i>Sfr</i> 13	1026	16	0.91	0.81	0.11	6	0.0000*	0.667
<i>Sfr</i> 22	1102	25	0.94	0.82	0.12	6	0.0010*	0.135
<i>Sfr</i> 34	1074	28	0.81	0.69	0.15	9	0.0010*	0.007
<i>Sfr</i> 55	930	35	0.96	0.74	0.23	13	0.0030*	0.002
<i>Sfr</i> 64	1039	9	0.83	0.76	0.09	2	0.0023	0.005
<i>Sfr</i> 90	895	14	0.63	0.66	0.04	0	0.0051	0.002
Mean		20	0.85	0.76	0.11		0.0020	

<sup>1</sup> Locus primers from Vadopalas & Bentzen (2000).

allelic richness was observed ( $P < 0.01$ ), with a greater number of alleles observed in southern sites (Table 1). Alternately, no significant heterogeneity in allelic richness or heterozygosity was observed among sites for *Sfr* ( $P > 0.05$ ). Similarly, less variation in  $F_{IS}$  was observed among sites than among loci for both species ( $F_{IS}$  among sites: 0.12–0.13 *Pab*, 0.08–0.13 *Sfr*;  $F_{IS}$  among loci 0.05–0.30 *Pab*, 0.03–0.23 *Sfr*). Despite the high degree of polymorphism displayed at all loci of both species, few private alleles (i.e., alleles observed in only one site) were observed. Out of 355 alleles for *Pab* observed over all loci, only 6 were private. For *Sfr*, 24 of the 265 alleles were private. Private alleles were not found in either species at frequencies above 0.03, nor were they found disproportionately in specific sites or regions.

For both species,  $F_{ST}$  calculated over all loci and all populations was significantly greater than zero ( $P < 0.01$ ); however, only the *Pab*  $F_{ST}$  was significant after jackknifing ( $P < 0.0004$ ). The global  $F_{ST}$  estimate for *Pab* ( $0.004 \pm 0.001$ ) was twice as high as that observed for *Sfr* ( $0.002 \pm 0.001$ ).  $F_{ST}$  values for single *Pab* loci ranged from 0.002–0.005, and were significantly greater than 0 for all eight loci ( $P < 0.05$ ) (Table 2). Alternately, for *Sfr*,  $F_{ST}$  values for single loci ranged from –0.001–0.006, and four of the seven loci (*Sfr* 34, 55, 64 and 90) were significantly greater than 0. When calculated on a pairwise basis, after bonferroni correction, over 85% of population comparisons were significant for *Pab*, versus only 10% for *Sfr* (Table 3).

The majority of the genetic variation in *Pab* was found within sites (99.5%), with only 0.4% partitioned among sites and 0.08% among regions. Sites represented a significant portion of the variation in the AMOVA (F-ratio = 1.88, df 12,16482,  $P = 0.03$ ), but permutations of models based on different regional configurations did not reveal any significant effects of region ( $P > 0.2$  in all models). However, in the neighbor-joining dendrogram, geoduck

samples generally clustered into four geographically-based units (Fig. 2A), and the mean  $F_{ST}$  values calculated among the four depicted regions (range of 0.003–0.005) were generally twice as high as those observed within regions (range of 0.002–0.003). The geographic cluster containing the three Queen Charlotte Island (QCI) samples, Gowgaia Bay, Hippa Island, and Tasu Sound, was the most distinctive. Although the two Washington samples, Point Heyer and Hood Canal, clustered together, they were fairly distinct from one another (significantly different at 3/8 loci), and were not highly differentiated from two of the East Coast of Vancouver Island (ECVI) sites, Marina Island and North Savory. However, they were well differentiated from all other sites on the BC coast. The remainder of the ECVI samples clustered together. The West Coast of Vancouver Island (WCVI) sites and single North Coast of Vancouver Island (NCVI) site formed the fourth cluster. In all, the distribution of genetic variation in *Pab* was consistent with an IBD model of structure ( $R^2 = 25.5$ ,  $P < 0.0001$ ; FSTAT), but it was clear from the low  $R^2$  value that all sources of variation were not accounted for by this model (Fig. 3A). An IBD model of structure was also supported using the IBD program of Bohonak (2002), but the  $R^2$  and  $P$  values were generally lower. A higher degree of significance was obtained when log values of genetic distance were used, and when the two Washington sites were removed ( $P < 0.002$  versus  $P < 0.023$ ). Alternately, IBD structure was not supported when the three QCI sites were removed ( $P > 0.35$ ).

Despite the fact that a lower percentage of population pairs were significantly different in *Sfr* versus *Pab*, hierarchical gene diversity analysis revealed a slightly greater amount of variation partitioned among sites than observed in *Pab* (1.24% vs. 0.48%). This difference is likely because of the higher number of private alleles observed in *Sfr*. Hence, sample site accounted for a significant portion of the variation in *Sfr* (F-ratio = 3.14, df 11,2415,



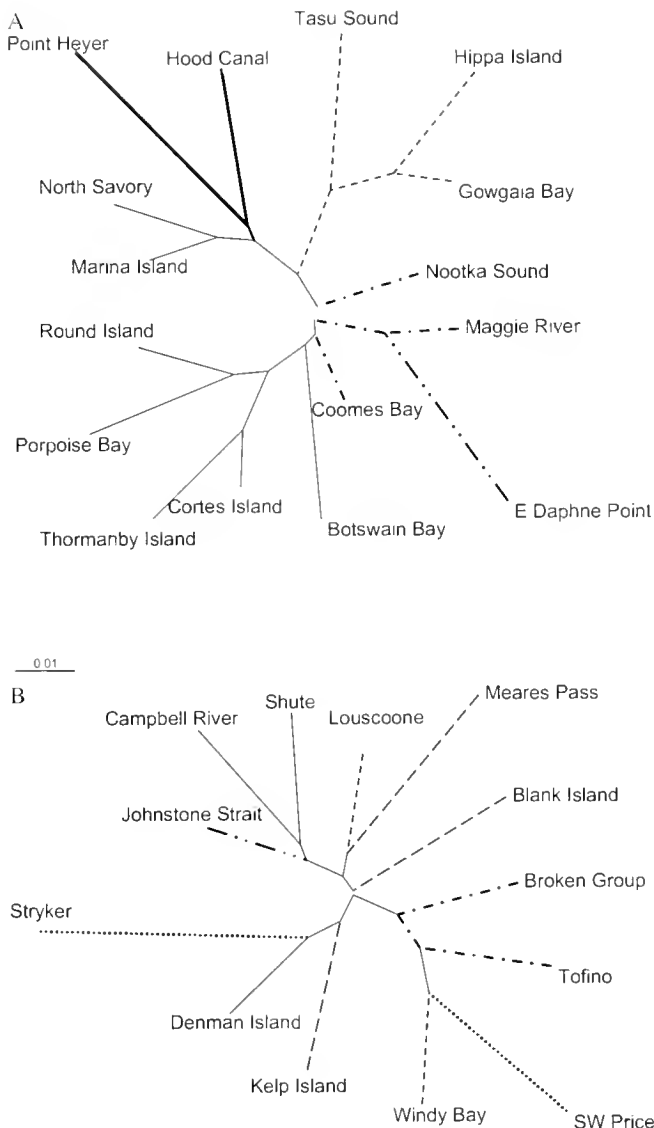


Figure 2. Neighbor-joining dendrogram based on Nei's (1978) genetic distances among A. *P. abrupta* and B. *S. franciscanus* collection sites. Regions are delineated through line type within the dendrogram, with thick solid line for Washington (*P. abrupta* only), thin solid line for ECVI, line-dot-line for WCVI, line-dot-dot-line for N-ECVI, short dashed line for QCI, dotted line for central coast (*S. franciscanus* only), and long dashed line for Alaska (*S. franciscanus* only).

$P = 0.0003$ ). However, no strong geographic clustering of *Sfr* samples was observed in the neighbor joining dendrogram (Fig. 2B), nor was regional structure supported in hierarchical gene diversity analysis (North/South regions, F-ratio = 1.44, df1,11,  $P = 0.26$ ). Given that the highest pairwise  $F_{ST}$  value (0.007;  $P = 0.01$ , not significant after bonferroni correction) was observed between the two north coast populations, South West Price Island and Stryker Island, that are only 40 km apart, it is not surprising

that an IBD model of structure was also not supported ( $R^2 = 1.07$ ,  $P = 0.36$ ; FSTAT; Fig. 3B).

## DISCUSSION

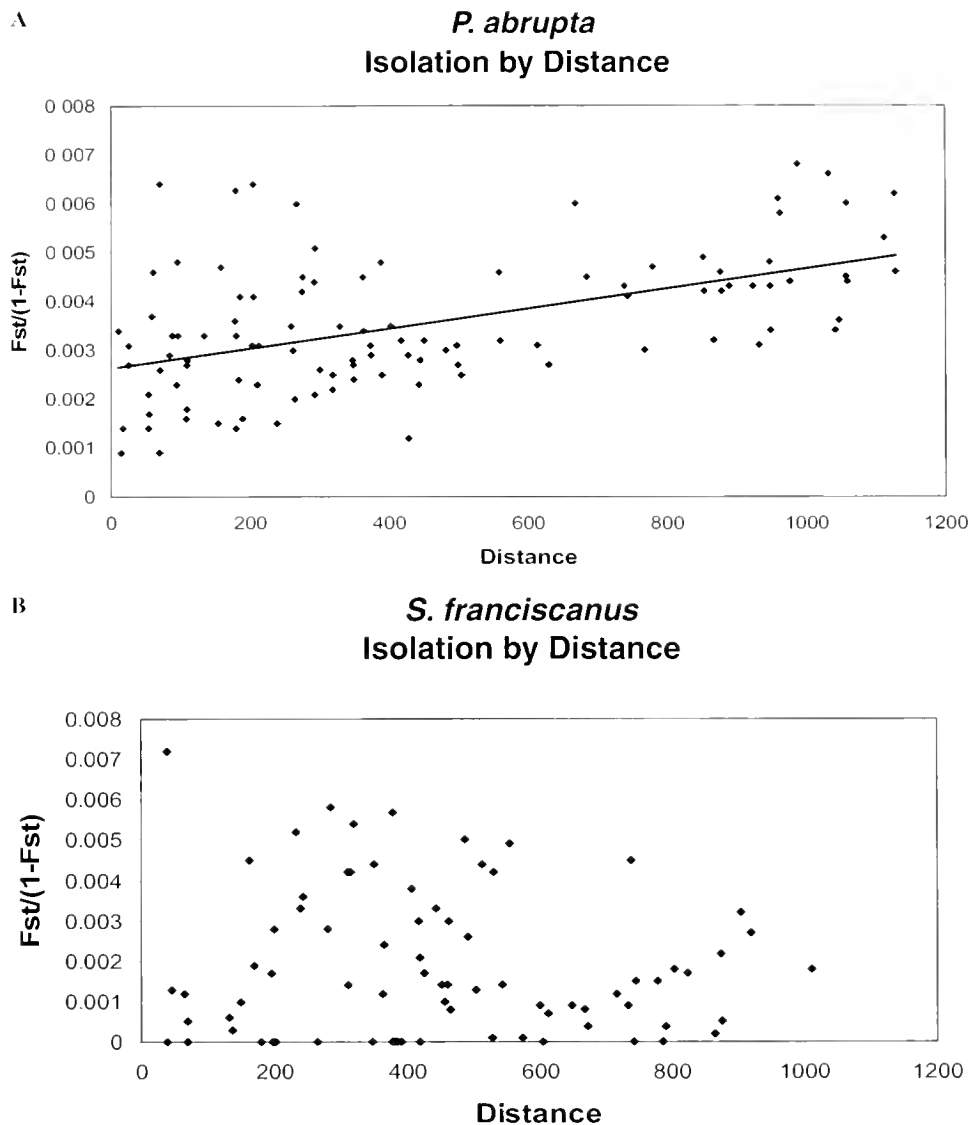
Current and potential culture operations for geoduck clams and red sea urchins in BC generally aim to enhance harvestable populations (Alan Campbell, pers. comm.). The degree of genetic risk to wild populations associated with these activities is largely influenced by: (1) the degree of assimilation of cultured releases into natural populations; (2) genetic differences between the natural and supplementation populations and (3) the degree of substructure of the natural population (Utter & Epifanio 2002). Our spatial analysis of microsatellite variation in *Pab* and *Sfr* provide the first view of substructure in natural populations of these species in BC, although additional sampling over time will be required to verify conclusions about stable population structure. In addition, data from this study provide a basis for the monitoring and evaluation activities recommended by Utter & Epifanio (2002).

For *Sfr* in BC, we found no evidence of a stepping stone pattern of connectedness (IBD model). Although the lack of geographically-based structure and "randomly" distributed private alleles could fit a model of chaotic genetic patchiness, more data focusing on genetic variation among settling cohorts would be required to provide strong support for this model. In general, our data do not refute the null hypothesis of genetic panmixia of *Sfr* within BC and SE Alaska.

Previous genetic studies of *Sfr* have focused on the southern portion of their range (Edmands et al. 1996, Debenham et al. 2000, Moberg & Burton 2000). Our results are consistent with these studies and with genetic studies of other sea urchin species. Generally, panmixia is not rejected at macrogeographic scales (Palumbi & Wilson 1990, Palumbi & Kessing 1991, Debenham et al. 2000, Addison & Hart 2004), but chaotic genetic patchiness is often observed at microgeographic scales (1–200 km) (Edmands et al. 1996, Flowers et al. 2002). None of the studies to date have supported an IBD model of structure. However, genetic structure caused by phylogeographic boundaries was observed in three species (Mladenov et al. 1997, Addison & Hart 2004).

Patterns of geographical genetic differentiation may reflect events in the distant past (thousands to millions of years ago) as much or more than ongoing patterns of dispersal (Brown et al. 2001, Hellberg et al. 2002). Hence, whereas the lack of genetic differentiation of *Sfr* within BC is consistent with significant gene flow over evolutionary time (thousands of years), recruitment data suggest that over ecological time (tens to hundreds of years), sites may not be as connected by larval dispersal as they appear (Ebert et al. 1994). Recruitment of *Sfr* is sporadic, with large pulses of recruitment occurring only every 3–5 y (ibid), and variation in the distribution of size classes among sites indicates high variability in recruitment over small spatial scales (0.5–8 km) (Sloan et al. 1987). These observations suggest that over ecological time scales, exchange among sites may be more limited than genetic analyses suggest.

*Pab* inhabits coastal and estuarine sites from California to Alaska and may occur in small numbers in southern Japan (Coan et al. 2000). *Pab* can be found in the low intertidal zone down to depths of 100 m (Goodwin & Pease 1989). We observed a higher level of spatial genetic structuring in *Pab* among the BC sites than reported among sites within Puget Sound and between Puget Sound and an Alaskan site by Vadopalas et al. (2004).



**Figure 3.** Isolation by distance in A, *P. abrupta* and B, *S. franciscanus*. Distance is the minimum waterway distance in km. The  $R^2$  for *P. abrupta* was 25.5, and was highly significant ( $P < 0.0001$ ). The  $R^2$  for *S. franciscanus* was 1.07 and was not sig ( $P = 0.36$ ).

Although different microsatellite loci were examined in the two studies, both included *Pab* 5 and *Pab* 6. The  $F_{ST}$  values for *Pab* 5 and *Pab* 6 were higher in BC (0.005 and 0.003, respectively) than in Puget Sound (0.001 and 0.0001, respectively), indicating greater genetic variability among BC than Puget Sound sites. Whereas the overall geographic range of the two studies was similar, the IBD structure resolved in our study was not supported in the Vadopalas et al. (2004) study. This discrepancy is likely because of differences in the sampling distribution of the two studies: most of the samples in Vadopalas' study were collected within 200 km of one another within the Puget Sound basin and only a single distant Alaskan sample was compared, whereas the samples in our study were more evenly distributed across a span of over 1,100 km, with an average pairwise distance among sites of 445 km. By combining the data from both studies, we can conclude that *Pab* exhibits panmixia at small spatial scales of 50–300 km, and stepping stone gene flow at intermediate scales of 500–1,000 km.

The only *Pab* sites that did not cluster regionally were Marina and Savory Islands, which are located adjacent to one another in

the Strait of Georgia (ECVI) but clustered with Puget Sound instead of other ECVI sites. Both of these sites were exposed to heavy commercial fishing in the past, and the Marina Island population was so depleted that in 1990 it was closed to fishing and became a research site (Campbell et al. 2004). In the mid 1990s Marina and Savory Islands underwent intensive reseedling efforts, with records indicating that broodstock were collected locally (ibid). However, seed from Puget Sound hatcheries was used in reseedling some west coast Vancouver Island sites at a similar time. Thus, the apparent genetic similarity of the Marina and Savory Island samples with Puget Sound samples observed in this study may indicate the introductions records for these two sites are not complete.

Genetic differentiation between rockfish species inhabiting sites in southern Puget Sound and Strait of Georgia/Strait of Juan de Fuca (collectively called the Georgia Basin) (Stout et al. 2001, Buonaccorsi et al. 2002) indicates that the shallow sill at Admiralty Inlet and complicated bathymetry within Puget Sound lead to larval retention within the Sound. For most species, insufficient sam-

pling has been conducted to define the precise location of the biogeographic boundary (Stout et al. 2001, Vadopalas 2003, Vadopalas et al. 2004). Although little differentiation was observed among most pairs of sites in Vadopalas et al. (2004), the sample from Juan de Fuca Strait was distinct from Puget Sound sites. Our study did not contain a sample from the Juan de Fuca Strait, but sites within Puget Sound were significantly differentiated from 6/8 ECVI sites and all sites located in other BC regions. Further, 16/21 comparisons between ECVI and WCVI were significant. Given the vastly different ecological environments between ECVI and WCVI (one wave swept, one sheltered), genetic exchange between these waters may be further limited because of adaptive constraints.

In northern BC, the marine biota of the QCI tend to be distinctive from southern coastal areas (Arndt & Smith 1998, Kyle & Boulding 2000, Withler et al. 2003) due either to the existence of historical refugia or to isolation of the QCI from the mainland by eddies in Hecate Strait (Briggs 1974, Allen & Smith 1988, Hetherington & Reid 2003, Di Lorenzo et al. 2005). The distinction of the QCI region was also relatively well supported for *Pab* in our study, and we hypothesize that oceanographic factors (i.e., disjunction between the California Current and the Alaska Gyre located just south of QCI) have limited recent migration of *Pab* between QCI and more southerly sites. Whereas we cannot discount completely the possibility that historic recolonization was also a factor in the generation of a genetically distinct QCI population, the lack of private alleles and statistical support for QCI as a distinct region in hierarchical diversity analysis suggests that the differences observed are not deep enough to have been derived through historical isolation. This is an important point because statistical support for the IBD structure in *Pab* was dependent on the inclusion of the QCI sites, and if historical events have influenced their relationships, then true stepping-stone gene flow is not necessarily occurring. However, future analyses using data from a broader geographic range of sites and markers could be used to test this hypothesis.

For *Pab*, we suggest the adoption of at least 3 management areas within BC (QCI, Georgia Strait, and west coast Vancouver

Island) and one for Puget Sound. This regional structure corresponds well with five general shellfish transfer zones proposed for BC by the Introductions and Transfer Committee (Dorothee Kieser, Fisheries and Oceans Canada). These zones are Haida Gwaii (our QCI), Georgia Strait, West Coast Vancouver Island, Queen Charlotte Strait (represented by a single sample in our study, E. Daphne Point) and the north/central coast (not sampled in our study). Additional *Pab* sampling from the Queen Charlotte Strait and north/central coasts of BC is required to examine population structure in these regions.

For *Sfr*, the genetic data provide no evidence of strong substructuring within BC waters but do not preclude the presence of geographically-based adaptive differences in growth and survival in the wild. We recommend that at least two management areas be identified within BC associated with the bifurcation of the Subarctic Current into the California and Alaska currents off the west coast of Vancouver Island, and the likely influence of these current systems on recruitment and productivity of marine organisms. *Sfr* culture in northern BC should be conducted with organisms originating within the Haida Gwaii and north/central coast shellfish transfer zones, whereas those in southern BC should be based on organisms originating from waters contiguous with Vancouver Island (i.e., Queen Charlotte Strait, Georgia Strait and west coast Vancouver Island zones).

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## SUBSTRATE EFFECTS ON SURVIVAL, GROWTH AND DISPERSAL OF JUVENILE SEA SCALLOP, *PLACOPECTEN MAGELLANICUS* (GMELIN 1791)

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**ABSTRACT** In the scope of expanding seeding operations of juvenile scallops (*Placopecten magellanicus*) around Îles-de-la-Madeleine (Quebec, Canada), this study assesses the influence of substrate on growth, survival, dispersal and predation rates in controlled experiments. Three substrates were tested: gravel, sand and sand with empty scallop shells (to investigate the feasibility of modifying the substrate as a management strategy). Dispersal rates were studied with a substrate choice experiment. Predation experiments with two major scallop predators, the rock crab *Cancer irroratus* and the sea star *Asterias vulgaris*, were carried out on different substrate types. No significant difference in growth was observed among substrates and no mortality was observed during the experiment. This suggests other factors, such as hydrodynamics, may be more important than substrate. The dispersal rate was significantly greater on sandy substrate, whereas gravel and sand-shells had similar results. Crab predation was lower on the more heterogeneous substrate (i.e., gravel) whereas, sea star predation was lower on sand. Modifying the natural sand substrate by adding scallop shells could improve seeding success because it diminishes dispersal and predation by crabs.

**KEY WORDS:** scallop, *Placopecten magellanicus*, seeding, mortality, growth, dispersal, substrate, predation, *Cancer irroratus*, *Asterias vulgaris*

### INTRODUCTION

To restore the overexploited population of sea scallop *Placopecten magellanicus* (Gmelin 1791) around Îles-de-la-Madeleine (Quebec, Canada), an experimental restocking program was launched in 1993. After the pilot phase, commercial seedings started in 2000 with the intent to seed 35–50 million young scallops annually. However, because of the expected expansion of the program, the availability of substrate suitable for growth, survival and retention of seeded individuals is likely to become an issue. Around the islands, 70% of the substrate in the scallop habitat depth range (9–55 m) is sandy.

Substrate characteristics influence scallop survival and growth. Whereas adult sea scallops are found on various substrates, gravel and pebbles are the most suitable (Thouzeau et al. 1991a, Stokesbury 2002, Kostylev et al. 2003). Fine particles such as silt decrease survival rate (Yamamoto 1957), and sandy substrate may increase juvenile mortality (Stokesbury & Himmelman 1996, Cliche et al. 1994). In natural conditions, fine inorganic material that is easily resuspended in the water column by currents or wave action is known to have a negative impact on growth in some scallop species (Gruffyd 1974, Bricelj & Malouf 1984). Emerson et al. (1994) demonstrated that high concentrations of seston inhibit sea scallop growth. Nevertheless, substrate heterogeneity has a positive effect on scallop growth. Turbulence created by a heterogeneous bottom may cause local seston accumulations by slowing down the current (Pilditch et al. 1998). A decreasing current speed may improve scallop filtering efficiency, because scallop growth is inhibited in strong current conditions (Bricelj & Shumway 1991, Wildish & Saulnier 1993).

Substrate type is also an important factor for scallop dispersal, as demonstrated by several authors (Winter & Hamilton 1985, Barbeau et al. 1996, Stokesbury & Himmelman 1996, Arsenault et al. 2000). Young individuals are active swimmers (Manuel & Dadswell 1991), but they also must attach themselves to the bot-

tom with a byssus (Hatcher et al. 1996), which reduces dispersal and limits the displacement out of a suitable substrate (Caddy 1972). Therefore, hard substrates possess characteristics that limit juvenile scallop displacement (Caddy 1972, Thouzeau et al. 1991b, Parson et al. 1992, Hatcher et al. 1996). It has been observed that dispersal rates and swimming activities of *Chlamys islandica* and *P. magellanicus* are higher on sand (Stokesbury & Himmelman 1996, Arsenault et al. 2000).

The major cause of scallop mortality on the bottom is known to be predation by rock crabs (*Cancer irroratus*, Say) and sea stars (*Asterias vulgaris*, Verrill) (Barbeau et al. 1994, Hatcher et al. 1996, Nadeau & Cliche 1998). Seeded scallops may have a better chance of survival on heterogeneous substrates: they are less exposed to predators because they can hide in crevasses. Wright (2002) showed that scallops preferentially settled on heterogeneous substrates like pebbles when they were alone or in the presence of crabs. In addition, increasing particle sediment size tended to decrease the predation rate of sea stars on small scallops (11–15 mm) but not crab predation (Wong & Barbeau 2003).

The main goal of this study is to determine juvenile *P. magellanicus* survival, growth and dispersal on 3 different substrates (sand, gravel and sand with scallop shells) in laboratory experiments. In addition, the predation rate by rock crab (*C. irroratus*) and sea star (*A. vulgaris*) in relation to the substrate type was examined.

### MATERIAL AND METHODS

Juvenile scallops came from the stock prepared for the 2002 commercial enhancement off Îles-de-la-Madeleine. They were kept in experimental tanks individually supplied with filtered circulating seawater at ambient salinity and temperature. The water passed through a pool filter loaded with sand-blast sand size # 0 and 1, diameter 0.4 mm to 2 mm. The flow rate was adjusted to allow the water in the tanks to be renewed twice daily. Therefore, current velocity in tanks was very low. Temperature was monitored daily from 2002/05–2002/11 and varied between 2.9°C and 23.2°C. Total suspended particulate matter in the water circuit was measured

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weekly ( $2.53 \pm 1.54 \text{ mg} \cdot \text{L}^{-1}$ , mean  $\pm$  SE,  $n = 100$ ). Light followed the natural photoperiod, with the intensity dimmed to simulate bottom luminosity.

#### Survival and Growth

Scallop growth and survival were studied on 3 substrate types: gravel (D50 [median particle size of the sample] =  $8400 \mu\text{m}$ ), sand (D50 =  $336.5 \mu\text{m}$ ) and sand with complete scallop shells (superior and inferior valves; shell height =  $113 \pm 2 \text{ mm}$ , mean  $\pm$  SE,  $n = 33$ ) covering about 25% of the tank bottom. Experiments lasted 88 days, from 2002/05/30–2002/08/26 and water temperature varied between  $9.8^\circ\text{C}$  and  $23.2^\circ\text{C}$ . Four replicates of each treatment were distributed randomly in twelve 160-L tanks ( $0.8 \text{ m}$  length  $\times$   $0.5 \text{ m}$  width  $\times$   $0.4 \text{ m}$  height) (Fig. 1a,b). Each tank represented one trial. Scallop ( $25.0 \pm 0.1 \text{ mm}$  shell height, mean  $\pm$  SE,  $n = 177$ ) were tagged a few days before seeding with Hallprint plastic tags ( $4 \text{ mm} \times 9 \text{ mm}$ ) fixed with Bostik cyanoacrylate adhesive glue. The plastic tags were also used to fix a monofilament nylon line on each scallop. In each tank, 15 scallops were tethered with a 12-cm monofilament nylon line tied with a solid glued knot onto a galvanized clamp hidden 5 cm in the substrate. This system was designed to avoid scallop settlement along the tank walls. Live scallops were counted once a week.

Growth rate was calculated as the size increment between the beginning and the end of the experiment.

#### Dispersal

Dispersal was assessed with a substrate choice experiment (M. Fr  chette, Maurice Lamontagne Institute, pers. comm.) in three 1,500-L tanks ( $2.4 \text{ m}$  length  $\times$   $1.2 \text{ m}$  width  $\times$   $0.6 \text{ m}$  height) (Fig. 1c,d). The tested substrate was laid in the middle of the tank, covering a surface of  $1.20 \text{ m} \times 0.60 \text{ m}$  ( $0.70 \text{ m}^2$ ) and surrounded by the reference substrate around the perimeter of the tank ( $0.70 \text{ m}^2$ ). In a preliminary experiment, conducted at the average temperature and with each substrate type, indicated random scallop displacements and no location preference was observed (pers. obs.). Three treatments were used: gravel as tested substrate with sand as reference, sand as tested substrate with gravel as reference and sand with 25% of the area covered with entire scallop shells (shell height =  $110 \pm 3 \text{ mm}$ , mean  $\pm$  SE,  $n = 21$ ) as tested substrate with gravel as reference. Thirty juvenile scallops ( $25 \text{ mm}$  shell height,  $n = 1075$ ) were seeded on the central zone from a basket maintained above the water surface. Each trial, carried out on the three treatments simultaneously, lasted 54 h; 12 replicates were done for each treatment. Scallops that stayed on the tested substrate were counted regularly (T0, T2, T4, T6, T8, T12, T24,

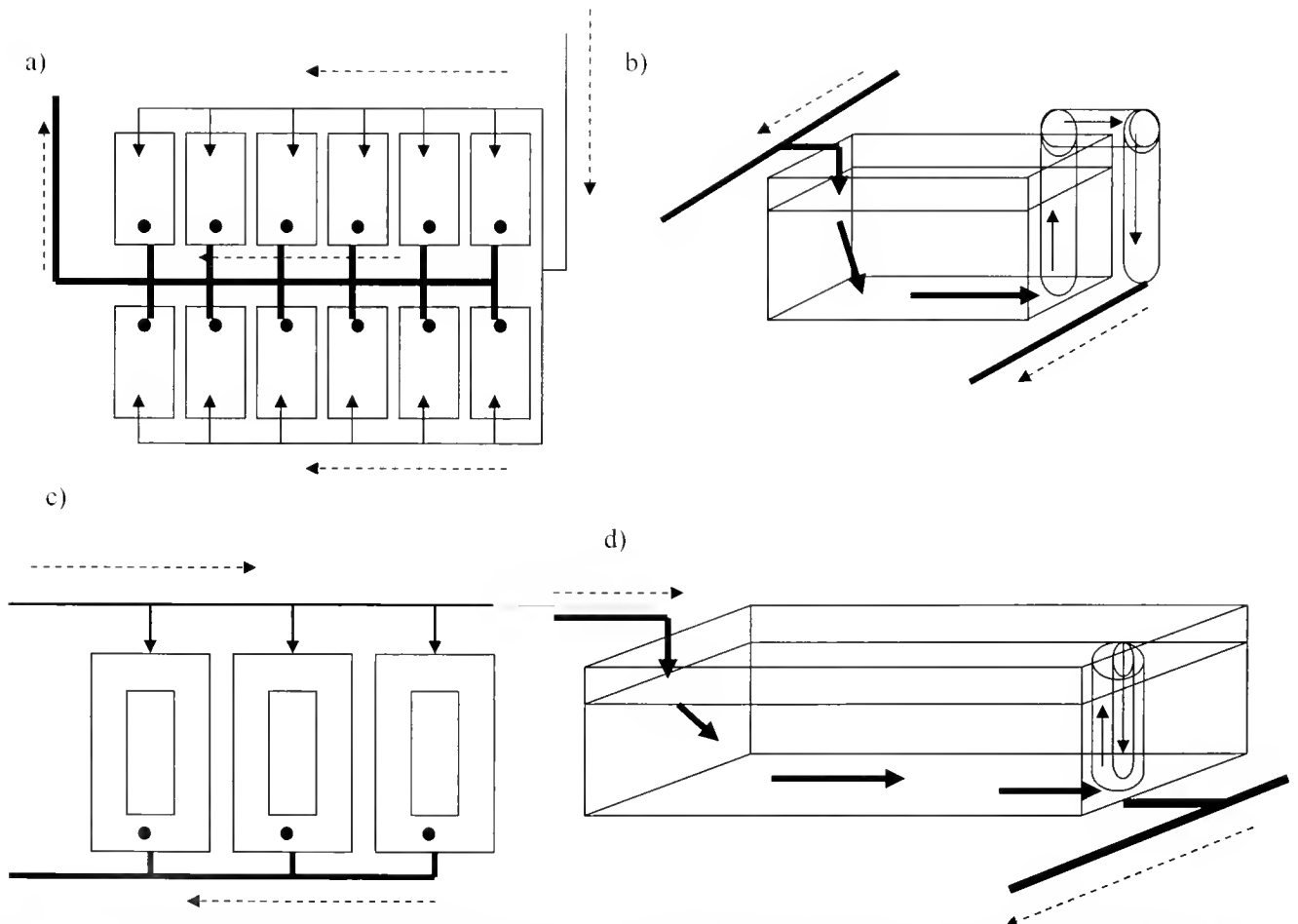


Figure 1. Set-up of tanks: (a) upper and (b) lateral views of the tanks system used for growth experiment, (c) upper and (d) lateral views of the tanks system used for dispersal and predation experiments.

T36, T48, T54 h). The experiment took place between May 24, 2002 and July 8, 2002, and water temperature varied from 8.6°C and 19.5°C. During the last seven replicates of the dispersal experiment, the position of the scallops (on the sand, or under or over shells) and the status (byssally attached or not) on the sand-shell substrate was noted.

### Predation

An experiment was conducted from September 17, 2002 to September 26, 2002 (water temperature from 13.3°C to 17.5°C) to evaluate predation on juvenile scallops by crabs and from October 15, 2002 to November 14, 2002 (water temperature from 2.9°C to 10.2°C) to evaluate predation by sea stars. Scallops used for these experiments were  $28.0 \pm 0.1$  mm shell height (mean  $\pm$  SE,  $n = 720$  scallops). Three 1500-L tanks (2.4 m length  $\times$  1.2 m width  $\times$  0.6 m height) (Fig. 1c,d) with a single substrate (gravel, sand or sand with scallop shells) were used. Tank size was expected to avoid limitations in escape responses because of tank's walls. Two predator species were considered: the rock crab, *Cancer irroratus* ( $92 \pm 1$  mm carapace width, mean  $\pm$  SE,  $n = 41$ ) and the sea star, *Asterias vulgaris* ( $74 \pm 2$  mm radius, mean  $\pm$  SE,  $n = 87$ ). Both predators were collected off Îles-de-la-Madeleine by a Digby-type scallop drag and acclimated to laboratory conditions for 1 month before the experiment. Predators were kept in perforated baskets (4 per basket) in a tank with filtered circulating seawater and fed twice a week with two mussels (*Mytilus edulis*) or two clams (*Mya arenaria*) per predator. Only male crabs were used to avoid sex-related biases. Predators were starved 48 h prior to the beginning of the experiment. During the crab experiments, two crabs were introduced into the tank 24 h after juvenile scallops had been added ( $n = 30$  per tank). Survivors were counted every 4 h until the end of the 12-h trial. For sea stars, four predators were used in a 96-h trial and their positions were noted at each observation time. Scallop survivors in each substrate treatment were counted every 24 h. Predator sizes and time periods were based on the results of preliminary experiments carried on for each predator (Nadeau et al. 1998). Four replicates of each substrate treatment were done for the crab and sea star experiments. During the trial, it was difficult to determine whether scallops were hidden or consumed by predators. Therefore, to avoid possible bias, scallops were removed at the end of each trial to count the number of survivors. The position and status (alive or dead) of scallops present on the sand-shell substrate were noted at each observation time.

We based our analysis of scallop reactions on the classification of their positions at the end of the trial: (1) in contact with a hard part of the substrate (shells or attached to a neighboring scallop); (2) in contact with shells only (attached or not; under or over a shell); (3) attached to hard part of substrate and (4) located under a shell but not necessarily attached to it (refuge use). Here we use "in contact" to describe scallops touching a hard substrate, whether they are attached or not. The first two categories identified the frequency at which scallops were using a hard surface in the presence of a predator and if shells were "preferred" or not. The third category determined the proportion of scallops in contact with a hard substrate but also byssally attached to it; this category defined a subset of scallops in the category one. The fourth category was used to define the proportion of scallop shells used as refuges; this category defined a subset of the category two. The proportion of hidden live scallops also served to compare the use of refuges in the presence of crabs and sea stars.

### Statistical Analysis

Statistical tests were done with Systat 10.2 software with a probability level ( $\alpha$ ) of 0.05. No statistical test was necessary for the analysis of survival data without predation because there were no mortalities. Growth data were analyzed using a 2-factor nested ANOVA with substrate (3 levels) as the fixed factor and tank (12 levels) as the random factor nested into the substrate. Normality was assessed using a 1-way Kolmogorov-Smirnov test and homogeneity of variances by a Levene test. The dispersal data were analyzed using 1-way ANOVAs with substrate (3 levels) as the fixed factor (1 per hour). The number of scallops present on the tested substrate (located in the middle of the tanks) at each observation time was used as the dependant variable. Normality was assessed using a 1-way Kolmogorov-Smirnov test and homogeneity of variances by a Cochran test (Critical C: 0.8709). One-way ANOVAs were used for crab and sea star predation data at the end of the experiment, with substrate as a factor (3 levels). This was done separately for each predator species. Normality was assessed by a 1-way Kolmogorov-Smirnov test and homogeneity of variances by a Levene test. A posthoc Tukey test was applied on the results to test specific differences when ANOVAs showed significant effects.

### RESULTS

No mortality was observed during the survival and growth experiments without predators. Growth did not differ significantly between substrates (ANOVA:  $F_{(2, 168)} = 1.62$ ,  $P = 0.852$ ). Growth rate was  $1.5 \pm 0.1 \times 10^{-1}$  mm  $\cdot$  d $^{-1}$  (mean  $\pm$  SE) on sand-shells,  $1.5 \pm 0.1 \times 10^{-1}$  mm  $\cdot$  d $^{-1}$  on gravel and  $1.5 \pm 0.1 \times 10^{-1}$  mm  $\cdot$  d $^{-1}$  on sand (Fig. 2). Tank had a significant effect on growth (ANOVA:  $F_{(9, 168)} = 10.07$ ,  $P < 0.001$ ). Growth differences were noted in relation to tank position in the water circuit (i.e., growth rate was lower in the middle than at the water inflow and outflow). This indicates that the system used may have created a bias as the slowest growth was observed in the middle section. All tanks were fed individually but the flow rate in tanks of the middle section was more variable and often difficult to adjust. However, that bias does not affect the general results.

Substrate had a significant effect on scallop dispersal (ANOVA:  $F_{(2, 33)} = 3.59$ – $18.78$ ,  $P < 0.05$ ). Weak dispersal was observed on gravel substrate (Fig. 3). The dispersal of scallops was

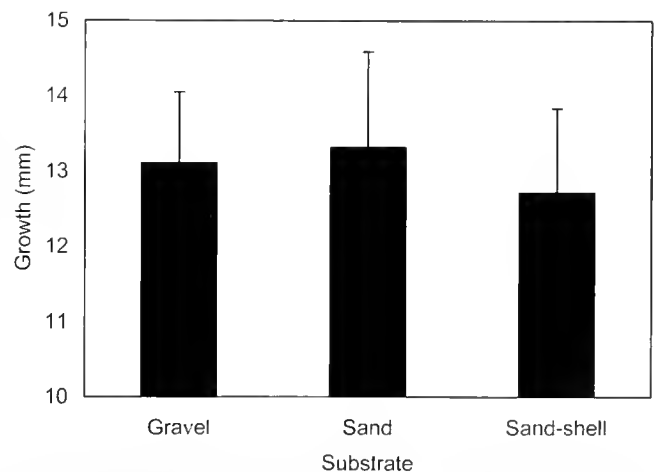


Figure 2. Mean growth (mm  $\pm$  standard error,  $n = 4$ ) of juvenile sea scallops on gravel, sand and sand-shell over 12 wk (88 days).

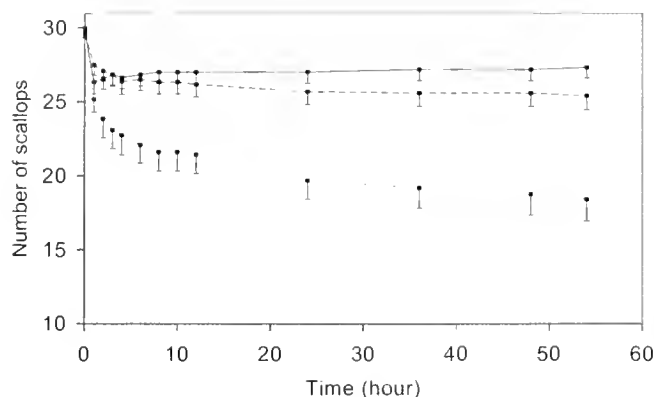


Figure 3. Mean hourly abundance (half standard error,  $n = 12$ ) of juvenile sea scallops remaining on each tested substrate (gravel: plain line; sand-shell: dashed line; sand: dotted line).

slightly higher on the sand-shell substrate than on gravel, but the difference was not statistically significant (posthoc Tukey test:  $P = 0.32$ ). Dispersal increased during the first 6 h on those two substrates and stabilized afterward, with only 10 and 17% of individuals moving off of the gravel and sand-shell substrates, respectively. Dispersal on sand was significantly higher than on the other two substrates. This difference was already significant by the second hour of the experiment and remained so until the end ( $F_{(2, 33)} = 3.59-18.78$ ,  $P < 0.05$ ). Within the first 6 h, 27% of the seeded scallops moved away from sand, increasing to 40% by the end for the trials. In all treatments, some individuals moved away from the reference substrate and went back to the tested substrate. Fourteen scallops returned to gravel, four to sand-shells and only two to sand.

On sand-shell substrate,  $76.8 \pm 3.9\%$  (mean  $\pm$  SE) of juvenile scallops were found in contact with hard parts of the substrate and the others were unattached. Of these scallops in contact with hard part of the substrate,  $71.6 \pm 9.3\%$  were found in contact with empty shells ( $70.0 \pm 12.9\%$  located under shells) and the others with a neighbor scallop. A proportion of  $40.1 \pm 8.66\%$  of individuals in contact with hard parts substrate were byssally attached to it.

After 12 h, crab predation was highest on sand (mean  $\pm$  SE,  $n = 7$ :  $9.3 \pm 2.6$  live scallops, i.e., 69% of scallops eaten) and lowest on gravel ( $22.8 \pm 1.5$  live scallops, 24% of scallops eaten) (Fig. 4). Survival on sand-shell was similar to that on gravel, with  $20.8 \pm 4.4$  live scallops (30% of scallops eaten). However, the

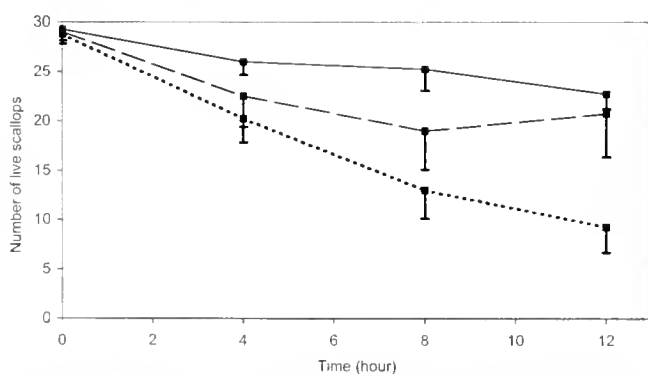


Figure 4. Mean number of scallops surviving crab predation at each observation time (half standard error,  $n = 7$ ) on the tested substrates (gravel: plain line; sand-shell: dashed line; sand: dotted line).

difference between the three treatments was not significant (ANOVA:  $F_{(2, 9)} = 3.24$ ,  $P = 0.087$ ). On sand-shell substrate, 20% to 40% of the scallops were hidden under shells. Crabs are fast-moving predators and required a short time for prey handling, leaving only shell fragments. When crabs were not moving, they were immobile on gravel (often near a corner of the tank) or buried in sand. Crabs often moved shells and seemed to have difficulty discriminating prey from nonprey items. Some scallops were so well hidden that counting was biased, causing an increase in the count of live scallops at the very end of the experiment. Thus, the final number is the most accurate because all scallops were removed and counted.

Contrary to crabs, sea star predation (Fig. 5) after 96 h was lowest on sand (mean  $\pm$  SE:  $22.8 \pm 1.9$  live scallops,  $n = 7$ ) and highest on gravel and sand-shells ( $17.8 \pm 2.3$  and  $17.0 \pm 1.1$  live scallops, respectively); however, the rates were not significantly different (ANOVA:  $F_{(2, 9)} = 1.63$ ,  $P = 0.248$ ). Gravel and sand-shell substrates showed a similar pattern: 41% and 43% of scallops were consumed, respectively, on these substrates after 96 h compared with an average of 24% on sand. The level of predation increased in the following order: sand < gravel  $\approx$  sand-shells. Sea stars are slow-moving predators, and the time required for prey handling is longer than for crabs; handling time per prey could last 24 h. Sea stars spent most of their time on the walls of the tanks. When they were not on the tank walls, they were found more often on the gravel substrate (average 1.6 sea stars per trial) and sand-shells (average 1.3 sea stars per trial) than on sand (average 0.8 sea star per trial).

The juveniles' use of scallop shells was different between the rock crab and sea star experiments. The proportion and number of live scallops observed under shells increased with time in the presence of crabs (Fig. 6a), whereas it was relatively stable in the presence of sea stars (Fig. 6b). On average,  $29.4 \pm 2.8\%$  (mean  $\pm$  SE,  $n = 4$ ) of the live scallops were hidden under shells in experiments with crabs, significantly higher than the average of  $10.4 \pm 1.5\%$  with sea stars (Mann-Whitney  $U$ -test,  $P < 0.0001$ ).

## DISCUSSION

Contrary to previous studies, substrate type in our experiment did not have any effect on juvenile scallop survival because no mortality (without predators) occurred. These differences can be attributed to experimental conditions, such as the low density and low current conditions in our tanks. Higher densities or faster

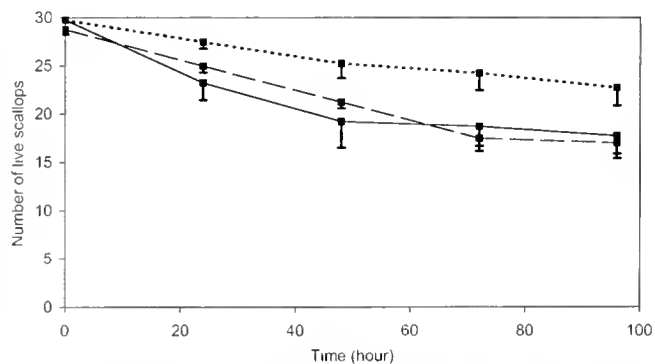


Figure 5. Mean number of scallops surviving sea star predation at each observation time (half standard error,  $n = 7$ ) on the tested substrates (gravel: plain line; sand-shell: dashed line; sand: dotted line).

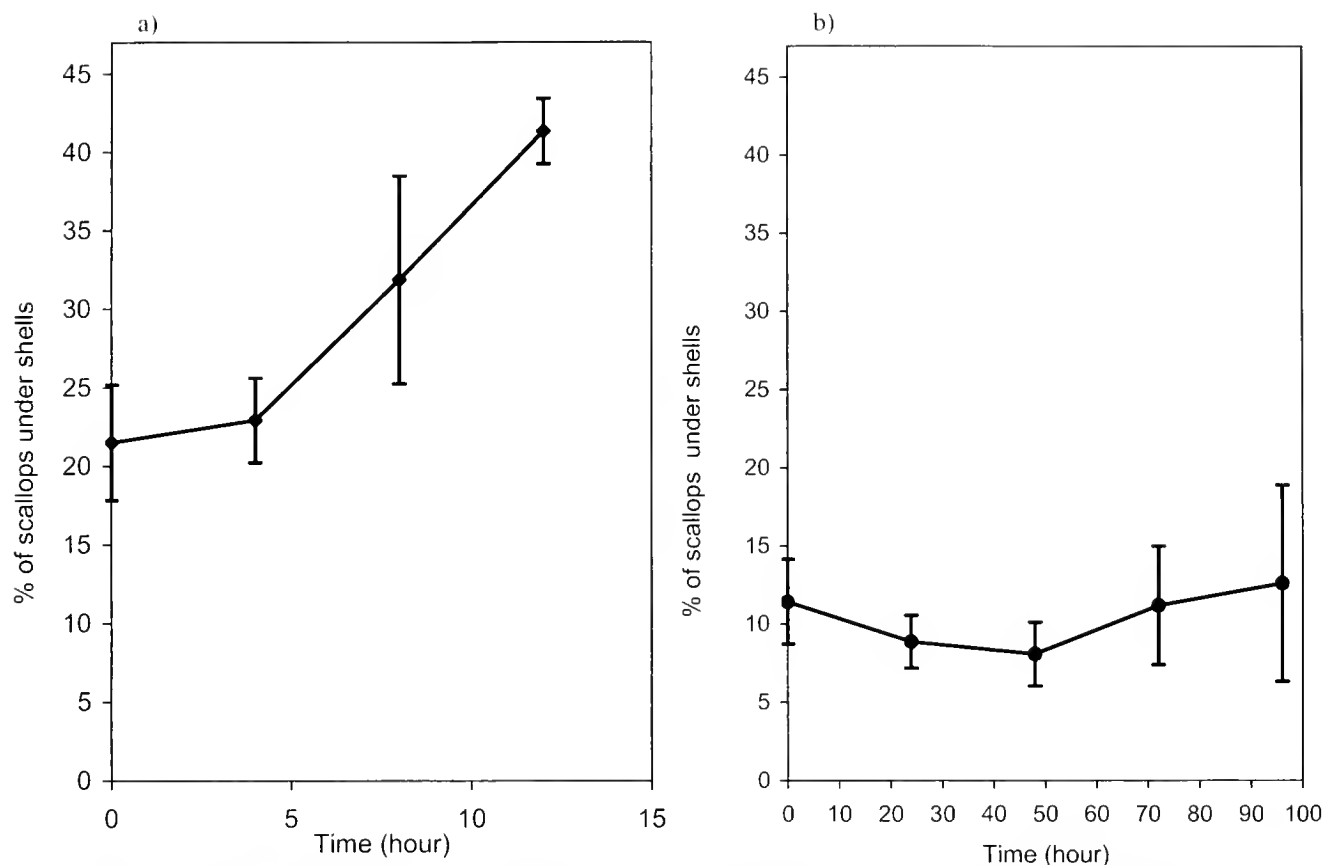


Figure 6. Percentage of live scallops found under shells (mean  $\pm$  SE,  $n = 4$ ) in the presence of (a) rock crabs and (b) sea stars.

water flow can favor resuspension of fine particles and increase mortality rates, as may be the case in the natural environment.

In our study, substrate type did not have any effect on growth rate. Scallop growth is known to be directly correlated with organic matter input (Kleinman et al. 1996, Pilditch & Grant 1999) and inversely correlated with inorganic matter (Cranford 1995). On heterogeneous substrates, like gravel or sand with shells, the topography may induce local accumulations of organic matter while reducing current velocity (Pilditch et al. 1998). Those accumulations and resulting increase in food availability would enhance scallop growth. The optimal current velocity for scallop filtering activity is  $10 \text{ cm} \cdot \text{s}^{-1}$  (Wildish & Saulnier 1993), whereas feeding is totally inhibited in strong currents (Wildish & Saulnier 1993). In our experiment, reduced flow and low organic matter input may have tempered the direct effect of the substrate on growth, suggesting that hydrodynamic conditions prevailing on the bottom may be more important than substrate alone.

As expected, dispersal was significantly different among substrate types, with the highest dispersal rate noted on sand. Adding scallop shells onto the sand induced a dispersal pattern similar to the one observed on gravel. Other studies found that dispersal was related to substrate type. In field experiments, Stokesbury and Himmelman (1995) observed less scallop dispersal when seeding occurred on gravelly and rocky bottoms than on sandy bottoms. The preferential use of heterogeneous substrate is related to juvenile scallop behavior (Hatcher et al. 1996): the juveniles in our study needed a suitable substrate for attachment. When scallops were seeded on sand, they swam until reaching a place to fix themselves, such as gravel or shells. Scallops on gravel spent less

time swimming and tended to remain on the same site. The addition of scallop shells to the sand increased the potential attachment sites, thus reducing scallop dispersal and making this substrate comparable to gravel. On the sand-shell substrate, 40% of the scallops were attached together in groups of two or three or attached to shells. Fr  chette (IML-MPO, pers. comm.) has observed some groups of two or three young scallops attached together on sand. This behavior was also noted on sand in this study, but it was unusual on the gravel substrate. However, the study by Stokesbury and Himmelman (1996) indicates that juvenile sea scallops disperse randomly. In this study, the high level of movements from sand to gravel cannot be attributed to a pure random dispersal. Present results should be completed with other studies to further document scallop's behavior on the various substrate arrangement.

Predation rates were not statistically significant between substrates. Temperature could have been a factor (Barbeau & Scheibling 1994b), but temperature changes in each predator experiment were not sufficiently high to account for those results. Because the number of replicates was low (four), individual behavior of predator could have masked any effect and, thus, a higher number of replicates (which was not feasible here because of technical constraints) could have shown more evident results. Even if not statistically demonstrated, trends, however are clear enough to be interpreted. Crab predation decreased with an increase in substrate heterogeneity, with a lower mortality being observed on gravel. On the sand-shell substrate in the presence of predators, as much as 40% of the live scallops were located under shells. A larger number of scallops used refuges on the sand-shell substrate in the crab experiment and this number increased with time. On sand, only

31% of scallops were still alive at the end of the experiment. There was less predation by crabs on the sand-shell substrate than sand. Crab predation is known to be mostly affected by the encounter rate (predator and prey density) because they are fast-moving and have a low prey handling time (Barbeau et al. 1994, Nadeau & Cliche 1998). Sponaugle and Lawton (1990) observed a higher consumption rate of *Mercenaria mercenaria* on sand than on sand-shell substrate by the crabs *Ovalipes ocellatus* (Herbst) and *Callinectes sapidus* (Rathbun) caused by a higher foraging time on the sand-shell substrate. During our experiment, crabs had difficulty discriminating prey items and nonprey items, as was also observed by Wong and Barbeau (2003). Adding shells to the bottom may increase crab foraging time, and scallop survival may be greater because of the decreasing encounter rate. Crabs are visual and chemodetecting predators and can move fast. The scallop escape response in our crab experiment was passive, scallops closed their valve, as has been observed by Barbeau and Scheibling (1994a and b). The presence of shells diminishes the encounter rate with scallops, and one can expect the same effect on crab predation in natural conditions.

In the presence of sea stars, scallop survival was greater on the sand substrate, unlike the experiment with crabs. Experiments on the sand-shell substrate showed survival values intermediate to survival on gravel and sand. When sea stars were not foraging, they moved along the walls of the tanks. Their foraging time on the gravel and sand-shell was twice as high as on sand, perhaps explaining the high scallop survival on sand. Sea stars are slow moving predators and can easily detect chemical signals of organic substances (Zafiriou 1972, Barbeau et al. 1994, Rochette et al. 1994, Gaymer et al. 2002). This explains why shells did not provide an adequate protection against sea stars, which were able to detect scallops in refuges. In addition, scallops have an efficient escape response from slow moving predators like sea stars, and juveniles have better swimming capacities to escape than adults (Barbeau et al. 1994). In fact, scallops did not use refuges as intensively as with crabs. Sea star predation rate is affected by a decrease, a weakness or a distortion of chemical signals (Rochette et al. 1994) and by the probability of capture on encounter (Barbeau et al. 1994). In natural habitats, shells can induce a distortion in the chemical signal in strong current condition and decrease encounter probability. The low current conditions in our experi-

mental tanks may explain why shells seemed to be less effective against sea stars than against crabs.

In our laboratory experiment, sand appeared to be a suitable substrate for scallop survival and growth that was similar to gravel and sand-shell substrate but less suitable for dispersal and against crab predation. Seeded scallops are more vulnerable to predation and need short-term protection against predators when they are released on the bottom. Scallop shells may increase the suitable area for byssus attachment and may provide some refuge from predators. The positive effect of adding shells to the bottom has already been shown. Guay (2003) found that the richness and the density of most common invertebrates increased on sand by adding scallop shells. That author also observed that 2 predators, *Leptasterias polaris* (Müller and Troschel) and *Hyas araneus* (Linnaeus), were attracted to sand and gravel enriched by shells, but that that attraction was more important on gravel. In field experiments, Pacheco and Stotz (2003) showed that settlement and survival of *Argopecten purpuratus* (Lamarck) was improved by adding crushed and empty shells to the bottom compared with natural bottoms (sand and mud). Thouzeau et al. (1991a) noted that the sand-shell (medium sand with quahog, scallop and surf-clam shells) portion of Georges Bank has a lower density of decapods and sea stars than coarse sediments like gravel and pebbles. The effect of substrate on survival, growth and dispersal of juvenile sea scallops was tested in the present controlled experiments. However, that effect may be different in the natural environment, where hydrodynamic processes and many other biotic and abiotic factors may influence the conditions. Attempts were made to transpose the experimental results to field experiments around Îles-de-la-Madeleine (Bourgeois 2004). Because technical biases occurred, those experiments were unable to provide useful data. Considering the promising results of this study, further research is required to better assess the feasibility of substrate modification to improve scallop seeding success.

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## BIVALVE CONTRIBUTION TO SHALLOW SANDY BOTTOM FOOD WEB OFF MAR DEL PLATA (ARGENTINA): INFERENCE FROM STOMACH CONTENTS AND STABLE ISOTOPE ANALYSIS

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**ABSTRACT** Two infaunal species, the purple clam *Amiantis purpurata* and the razor clam *Solen tehuatlchus*, are common species in the 15–20 m sandy bottom sediments between southern Brazil and central Argentina. Both species are food sources for the coastal food web, but the extent of their contribution to this food web is still unknown. Based on stomach content analysis and  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  stable isotope signatures we explored the position of these clams in the food web. Stomach content shows that the ray *Sympterygia bonaparteri* and the white croaker *Micropogonias furnieri* prey on entire *A. purpurata* and *S. tehuatlchus*. The stable isotopic analysis confirms this result. The elephant fish *Callorhynchus callorhynchus* and the Brazilian codling *Urophycis brasiliensis* also show values of C and N consistent with those expected from species that are preying on *A. purpurata*. Based on the prediction from the isotopic analysis the clam *A. purpurata* is a food source for the shrimps *Artemesia longinaris*, *Pleoticus muelleri*, the anemone *Antholoba achates* and the gastropod *Buccinanops monilifer*. The gastropods *Adelomelon brasiliana*, *Olivancillaria deshayesiana*, *Olivancillaria urceus* and *Zidona dufresnei* also show evidence of consuming *A. purpurata* but with contribution from other species with heavier C content. According to complementary results the razor clam *Solen tehuatlchus* may be this other species. The stable isotopic analysis shows that both clam species are at the base of the consumers in the food web. *A. purpurata* showed lighter C mark than *S. tehuatlchus*, but the N isotopic mark showed higher value but still being within the same trophic level.

**KEY WORDS:** food web, bivalves, fish, crustaceans, gastropods, SW Atlantic

### INTRODUCTION

Two infaunal bivalves, the purple clam *Amiantis purpurata* (Lamarck, 1818) and the razor clam *Solen tehuatlchus* (D'Orbigny 1843), are common species in the coastal ( $\leq 20$ -m depth) sandy bottom of the SW Atlantic. The purple clam is distributed from Espírito Santo, Brazil (20°S) to the Golfo San Matías, Argentina (42°S) (Carcelles 1944, de Castellanos 1967, Scarabino 1977) occurring in high-density patches (maximum abundance: 632 clams  $\text{m}^{-2}$ ) at depths ranging from 10–18 m dominated by 1- or 2-year-classes (Morsan 2003). The razor clam *S. tehuatlchus* ( $\leq 61$ -mm length) shows similar distribution, from Río de Janeiro, Brazil (22°54'S) to Bahía Blanca, Argentina (38°44'S) (Rios 1994, Capitoli 1997). However, the abundance of this species is not truly reflected in the literature, probably because of their deep burrowing habits. There is evidence that both species are important food sources for a number of species in shallow coastal areas but the extent of their contribution to the food web is still unknown.

Several predators have been described for these species based on gut content analysis (Olivier et al. 1968, Radonic 1997). For example, siphon tips of *A. purpurata* are the main food item of the electric ray *Discopyge tschudii*, Heckel 1846 (Arrighetti et al. 2005), which is the regionally most abundant torpediniform species. Also, entire clams are preyed by the fish *Pogonias cromis* (Linnaeus, 1766) (García & Gianuca 1997). However, gut content analysis is limited and may be biased toward larger species that can hold the bivalves for longer time (see Sutela & Huusko 2000, Hyslop 1980) and toward food items recently consumed; moreover, this method cannot provide information on the rate of ingestion and regarding long-term assimilation (Creach et al. 1997).

As an alternative, measurement of stable isotope ratios has

become widely used to define relationships between consumers and their food sources (Peterson & Fry 1987, Michener & Schell 1994, Cabana & Rasmussen 1994), and they have been applied to study foraging, migration and other life history phenomena (Hesslein et al. 1991, Alisauskas & Hobson 1993, Walker et al. 1999, Best & Schell 1996, Hansson et al. 1997, Kline et al. 1998, Griffin & Valiela 2001, McGinnis & Emslie 2001). Stable isotopes are relatively consistently fractionated by biological and physical processes as they pass through food webs becoming heavier by 3‰ to 4‰ for N and by 1‰ to 2‰ for C with each trophic transfer (Peterson & Fry 1987, Cabana & Rasmussen 1994). This fractionation from potential food sources to tissues in consumers has been used to identify trophic relationships. Trophic relationships between motile consumers like fishes and their food sources may depend on size of consumers and foraging patterns. Larger animals are able to feed higher in the food web and often acquire heavier isotopic signatures in their tissues (France et al. 1998, Harvey et al. 2002). Therefore, combining isotope analysis with gut contents can provide a better understanding of trophic pathways, given that some items could be ingested but not assimilated. Also, isotope analysis can provide information on those organisms in which it is not possible to evaluate gut content.

In this study, we used stomach content analysis whenever possible and  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  stable isotope signatures to define the position of the purple clam *Amiantis purpurata* and the razor clam *Solen tehuatlchus* in the food web of the shallow northern Argentinean coastal ecosystem.

### MATERIAL AND METHODS

#### Sampling and Study Areas

The study was performed at the coastal area of northern Argentina (38°20'S) between April 2002 and December 2002. This

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area is dominated by sandy and sandy-silty bottoms (Cortezzi in Olivier et al. 1968). The community studied is typical of a temperate marine environment where few taxa are dominant over a large number of occasional species. The low diversity of the infaunal assemblages has been described (Olivier et al. 1968, Scelzo et al. 2002). Samples of the two bivalves and of the most representative and abundant organisms coexisting with them were obtained from the study area with bottom trawls using a 2-cm cup net from water depth of 10–18 m. The species sampled were the fishes *Urophycis brasiliensis* (Kaup 1858), *Cynoscion striatus* (Cuvier 1829), *Sympterygia bonapartei* (Müller & Henle 1841), *Micropogonias furnieri* (Desmarest 1823) and *Callorhynchus callorhynchus* (Meuschen 1778); the gastropods *Buccinanops monilifer* (Kiener 1834) *Adelomelon brasiliana* (Lamarck 1811), *Olivancillaria deshayesiana* (Duclos 1857), *Olivancillaria urceus* (Roding 1798), and *Zidona dufresnei* (Donovan 1823); the anemone *Antholoba achates* (Drayton 1984) and the shrimps *Artemesia longinaris* (Bate 1888) and *Pleoticus muelleri* (Bate 1888).

#### Stomach Content Analysis

The stomach of 15 *Urophycis brasiliensis*, 35 *Cynoscion striatus*, 11 *Sympterygia bonapartei* and 42 *Micropogonias furnieri* were injected aboard with 10% formaline to stop the digestive process. In the laboratory, the total length of each fish was measured to the nearest mm. To analyze the stomach contents, the digestive tract anterior to the intestine was removed and preserved in 10% formaline. Stomach contents were observed under a dissecting microscope. Additional information was made on the gastro-vascular cavity of *Antholoba achates* and its associated snail *Adelomelon brasiliana*. This information was complemented by reported information from the same area (i.e., Olivier et al. 1968, Radonic 1997).

#### Stable Isotopes Analysis

Samples of white dorsal muscle of all fish species were removed and frozen for stable isotopic analysis. Samples were also

obtained from the epibenthic fauna (5 pools of 5 individuals each) for the same analysis. All these samples were dried at 60°C, tissues were ground using a mortar and pestle, and all samples were sent to the University of California-Davis Stable Isotope Facility to determine  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  signatures by mass spectrometry.

## RESULTS AND DISCUSSION

Stable isotope analysis shows three trophic levels based on expected N fractionation (3.4‰). The two bivalves *Solen techuelchus* and *Aniantis purpurata* are at the base of the food web, with  $\delta^{15}\text{N}$  values between 10.9‰ and 12.3‰. *S. techuelchus* has  $\delta^{13}\text{C}$  values more depleted than *A. purpurata* but similar N isotope ratios. The fishes *Callorhynchus callorhynchus* and *Cynoscion striatus* and the squid *Loligo gahi* (d'Orbigny 1835) are in the upper trophic level with  $\delta^{15}\text{N}$  values between 16.7‰ and 17.5‰. In our study system, stomach content analysis can only be reliable with fishes and anemones. Based on information previously reported and our sampling (Table 1), the ray *Sympterygia bonapartei* prey on siphons of *Aniantis purpurata* and *S. bonapartei*. A similar result was previously found for other ray species, the electric ray *Discopyge tschudii* (Arrighetti et al. 2005). *S. bonapartei* showed C and N isotopic values expected from a clam diet, but N isotope ratios showed high variability and could be the result of a mixed diet of both clam species and some contribution of *Artemesia longinaris* (see Fig. 1, Table 1).

Stomach contents showed that the white croaker *Micropogonias furnieri* prey on entire individuals of *A. purpurata* and *Solen techuelchus* (Table 1). This fish species showed different isotopic values between juveniles (<30 cm in length) and big individuals (Fig. 1), suggesting a shift in diet during their life cycle. Juveniles have values more depleted in C suggesting a food source other than clams, probably crustaceans or polychaetes (Olivier et al. 1968, Radonic 1997 and stomach content results). Adults, however, showed values that suggest an influence of clams. This shift in diet between sizes for this species was found for other sites (Olivier et al. 1968).

TABLE 1.  
Biomass of the prey items identified in the stomach content analysis (%)

Prey items	<i>C. striatus</i>			<i>U. brasiliensis</i>		<i>S. bonapartei</i>		<i>M. furnieri</i>		
	Olivier et al. 1968	Radonic 1997	Ours	Olivier et al. 1968	Ours	Olivier et al. 1968	Ours	Olivier et al. 1968	Radonic 1997	Ours
Crustacea										
<i>Artemesia longinaris</i>	5		17.5	46.7	6	21.6	7	22.4		
<i>Pleoticus muelleri</i>	0.6		0.5	5.1	1	4.2	10			
Other	93	77	69.2	43.8	75	62	71	57.2	45	37.3
Polychaete	1.2	1	2.7			5.4		6.1	13	
Osteichthyes	2	2		4.4	18	6.8		6.1	1	
Mollusca										
Bivalve										
<i>Aniantis purpurata</i>							4			34.7
<i>Solen techuelchus</i>							3			28
Other								6.1		
Gastropoda										
<i>Buccinanops monilifer</i>								2		
Cephalopoda										
<i>Loligo gahi</i>									41	
Cnidaria		3								
Chaetognata		17								
Others			8.1							

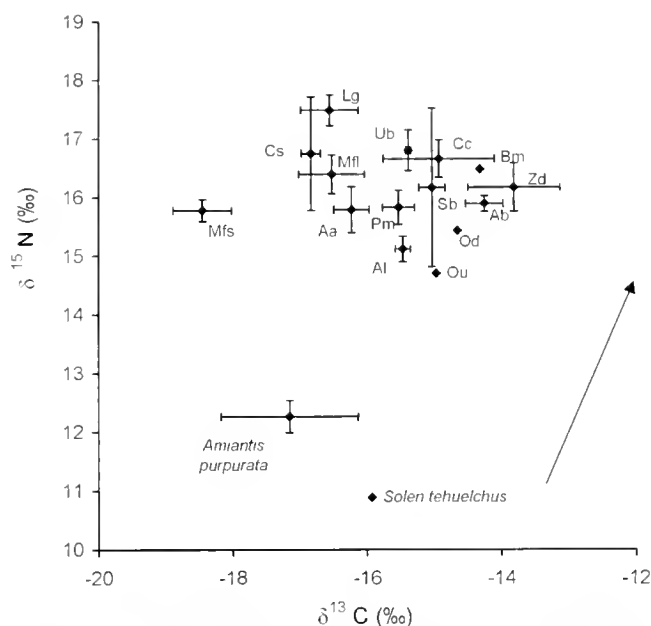


Figure 1.  $\delta^{15}\text{N}$  versus  $\delta^{13}\text{C}$  (mean  $\pm$  1 SE) for the subtidal sandy bottom food web. *Olivancillaria urceus* (Ou), *Olivancillaria deshayesiana* (Od), shrimp *Artemesia longinaris* (Al), anemone *Antholoba achates* (Aa), white croaker *Micropogonias furnieri* small (Mfs) and large (Mll), shrimp *Pleoticus muelleri* (Pm), gastropod *Adelomelon brasiliense* (Ab), gastropod *Zidona dufresnei* (Zd), gastropod *Buccinanops monilifer* (Bm), ray *Sympterygia bonapartei* (Sb), Brazilian codling *Urophycis brasiliensis* (Ub), elephant fish *Callorhynchus callorhynchus* (Cc), striped weakfish *Cynoscion striatus* (Cs), squid *Loligo gahi* (Lg). The arrow shows expected enrichment in one trophic level.

The Brazilian codling *Urophycis brasiliensis*, and the striped weakfish *Cynoscion striatus* (Olivier et al. 1968, Radonic 1997 and our results; Table 1), did not show clams in their stomachs. Carbon isotope ratios of the striped weakfish *C. striatus* showed no evidence of preying on these clams (Fig. 1). The elephant fish *Callorhynchus callorhynchus* and the Brazilian codling *U. brasiliensis* show values of C consistent with those expected from species that are preying on the purple clam *A. purpurata*, but higher N signatures. This could be the result of a mix diet of items in more than one trophic level for example; in this case they could be feeding on shrimps (as seen by stomach contents) and having some contribution of clams.

For invertebrates it is much more difficult, or just impossible, to recognize items in the stomach content by microscopical analysis. Based on the prediction from the isotopic analysis the clam *Amiantis purpurata* is a food source for the shrimps *Artemesia*

*longinaris* and *Pleoticus muelleri*, and the gastropod *Buccinanops monilifer*. However, the gastropods *Adelomelon brasiliense*, *Olivancillaria deshayesiana*, *Olivancillaria urceus* and *Zidona dufresnei* also show evidence of consuming the purple clam but with contribution from the razor clam *Solen tehuelchus* (Fig. 1).

One of the few invertebrate species where items in the stomach content can be recognized is the anemone *Antholoba achates* (see Acuña et al. 2003). Our results show that 60% of the weight was *Amiantis purpurata*, whereas the other species contributed with much lower values (*Solen tehuelchus* 5%, *Encope emarginata* 11.5%, *Artemesia longinaris* 11.5%, *Olivancillaria uretai*, *O. Urceus* and *Buccinanops moniliferum* 10%, the hermit crab *Pagurus excilis* 0.6%, *Leucipa patagonica* 0.4% and polychaetes 1.1%). Isotope C and N also showed values expected for a diet mainly on *A. purpurata*. Interestingly, this anemone lives epibiotic on the gastropod *Adelomelon brasiliense* (see Acuña et al. 2003). *Adelomelon brasiliense* is a common inhabitant of the studied area, which preys on *Amiantis purpurata* and *Solen tehuelchus* of all sizes (Cledón 2005). These snails are long-lived and can reach over 200 mm in length (Cledón et al. 2005), which means a high predatory pressure for the bivalves, because no size is out of predation risk and their burrowing in the sediment is not always effective, the snails can also do that (Cledón 2005). The anemone and the snail are at the same trophic level (i.e., same *n* values) but C isotope ratios suggest that their food sources are somewhat different (i.e., the C mark differ). Whereas the anemone showed  $\delta^{13}\text{C}$  values expected of a predator feeding on *A. purpurata* and no on *S. tehuelchus*, the gastropod *A. brasiliense* showed  $\delta^{13}\text{C}$  values suggesting a diet that incorporates both bivalves at similar proportion. The sea anemone and the snail, although highly associated, do not show evidence of large diet overlap. This fact could be related to the burrowing activity of *A. brasiliense*, whereas *A. achates* would remain in contact with the upper sediment levels (Arrighetti et al. 2004).

In conclusion, the purple clam *Amiantis purpurata* and the razor clam *Solen tehuelchus* are species clearly located at the bottom of the food web. These two species contribute to the diet of some gastropods and sea anemones and to some economically important fish.

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## MORPHOLOGICAL, STRUCTURAL AND FUNCTIONAL CHARACTERISTICS OF THE HEMOCYTES OF THE OYSTER, *CRASSOSTREA ARIAKENSIS*

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**ABSTRACT** Light and electron microscopical studies were carried out to characterize the hemocytes of the oyster, *Crassostrea ariakensis*. Three types of hemocytes were recognized: granulocytes, large hyalinocytes and small hyalinocytes. The large hyalinocytes and small hyalinocytes were agranular cells. The large hyalinocytes presented thin cytoplasm and high nucleus: cytoplasm ratios. The small hyalinocytes were the most homogeneous in shape and showed round or oval. They have the highest nucleus: cytoplasm ratios and contained very thin cytoplasm. The granulocytes showed abundant cytoplasm and low nucleus: cytoplasm ratios. At the ultra-structural level, the large hyalinocytes cytoplasm contained a variable number of mitochondria, Golgi complex and endoplasmic reticulum. They showed a total absence of cytoplasmic granules or a few small electron-lucid vesicles of different sizes. The granulocytes showed more polymorphic than the agranular cells, with numerous pseudopodia sprouting off their surface. The granulocytes showed similar organelles, but on the contrary they had abundant electron-dense particles or electron-lucent granules in the cell cytoplasm. The small hyalinocytes presented a total absence of cytoplasmic granules and few organelles and only one or two mitochondria were sometimes observed in the cytoplasm. Total hemocyte counts gave a mean ( $\pm$ SE) concentration of  $2.06 \pm 0.20 \times 10^7$  cells mL<sup>-1</sup> of hemolymph. Differential hemocyte counts identified the granulocytes as the predominant cell type, followed by the large hyalinocytes and the small hyalinocytes. The percentages of the three cell types in the hemocyte population of *C. ariakensis* were 68.4%, 21.6% and 9.0%, respectively. The quantitative study of phagocytosis showed that the granulocytes were phagocytic cells and the agranular cells showed a limited phagocytic ability. After the separation in Percoll density gradient, the granulocytes were separated from agranulocytes and pure granulocytes were obtained. However, the separation of different agranular cell types (large hyalinocytes and small hyalinocytes) was not achieved by Percoll or Ficoll gradient centrifugation. Our study provides a morphofunctional basis for the cellular defense mechanisms in *C. ariakensis*.

**KEY WORDS:** *Crassostrea ariakensis*, hemocytes, granulocytes, hyalinocytes, phagocytosis, separation

### INTRODUCTION

Hemocytes of bivalve molluscs play an important and central role in the internal defense, and are known to be involved in other processes like wound and shell repair, nutrient digestion, transport and excretion (Cheng 1981). There have been many studies on the morphology, structure, function and classification of hemocytes in bivalves. The most important reviews of the various morphofunctional aspects of the hemocytes of the whole Mollusca phylum are those of Cheng (1981) and Hine (1999), who identified 2 fundamental hemocyte types in bivalve hemolymph: granulocytes and hyalinocytes (or agranulocytes). The presence of these two types was confirmed in *Mya arenaria* (Huffman & Tripp 1982), *Mytilus edulis* (Pipe 1990), *Mytilus galloprovincialis* (Cajarville & Pal 1995, Carballal et al. 1997a, 1997b), *Mercenaria mercenaria* (Tripp 1992), *Crassostrea virginica* (Ford et al. 1994) and *Ruditapes decussatus* (Lopez et al. 1997a). However, the classification schedules were so varied that three, four or even more morphologically different populations have been proposed by authors for various bivalve species (Moore & Lowe 1977, Cheng & Downs 1988, Hine & Wesney 1994, Nakayama et al. 1997).

The oyster, *Crassostrea ariakensis*, is one of the most important commercial mollusk species in China, whose natural range is from the south China coast through Southeast Asia to the western coast of the Indian subcontinent. In China, the culture of *C. ariakensis* has a long history in the Pearl River Delta, Guangdong Province. In recent years, the mass mortality has occurred in cultivated oysters with a great loss. Some studies revealed that the oysters were infected by the pathogen, a Rickettsia-like organism

(RLO) (Wu & Pan 2000, Sun & Wu 2004). However, no systematic studies have been carried out to investigate the morphology, structure, function and classification of hemocytes of *C. ariakensis*. A better understanding of the defense mechanisms in this bivalve species may lead to practical approaches to control RLOs and to avoid mass damage. Here we report the systematic morphological and structural characteristics of the hemocytes in the hemolymph of the oysters. Phagocytosis and separation of hemocytes by discontinuous density gradient centrifugation were also studied. Our study provides a morphofunctional basis for the cellular defense mechanisms in *C. ariakensis*.

### MATERIALS AND METHODS

#### Hemolymph Collection

The oysters, *C. ariakensis* (length: 6.0–9.8 cm; width: 4.5–6.6 cm; height: 9.0–14.0 cm) were collected from Hailing Bay in Yangxi County of Guangdong Province, China. Approximately 0.5–1 mL of hemolymph was extracted from the posterior adductor muscle of each animal using a 25-gauge needle into an equal volume of either Baker formol–calcium fixative (4% formaldehyde, 2% sodium chloride, 1% calcium acetate) or 0.05 M Tris–HCl buffer (TBS; pH 7.6, containing 2% sodium chloride), or into an equal volume of EM fixative (2% formaldehyde, 2.5% glutaraldehyde, 2% NaCl, 2 mM calcium chloride in 0.2 M cacodylate buffer, pH 7.4), as appropriate. A minimum of 20 samples was used for each immune parameter investigated.

#### Light Microscopy Observation

To characterize the hemocytes, the staining technique with Hemacolor kit (Merck) on hemolymph smears was carried out to

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distinguish the hyalinocytes and granulocytes cells. According to the presence or absence of granules in the cytoplasm of the cells, differential hemocyte counts were carried out on Hemacolor smears, and the percentages of different cell types were calculated.

Total hemocyte counts were carried out with an improved Neubauer hemocytometer using Baker's fixed hemolymph samples. Mean cell parameters (cell diameters and nuclear diameter) were calculated by measuring each cell type on hemacolor stained smears using Motic images system.

#### Transmission Electron Microscopy

The suspension of hemolymph fixed with transmission electron microscopy (TEM) fixative solution was centrifuged ( $\times 750$  g, 10 min). The pellets were washed in Pipes buffer with sucrose for 2 h at 4°C and post fixed in 1% osmium tetroxide in Pipes buffer for 75 min at 4°C. After being washed in Pipes buffer, the cells were embedded in 1.5% agar at 40°C and quickly centrifuged ( $\times 1,700$  g, 5 min). Then, the pellets were dehydrated and embedded in Epon. Ultrathin sections, (50–70 nm), were stained with uranyl acetate and lead citrate and examined in a TEM JEOL 100CX11.

#### Scanning Electron Microscopy

Fresh hemolymph was fixed with glutaraldehyde at 2% (v/v) in Millonig 0.2 M; pH 7.3 buffer solution, washed in buffer, post-fixed with osmium tetraoxide at 1% (p/v) and placed in dehydration slides with ethanol and amilum acetate, followed by a critical point desiccation process with CO<sub>2</sub> and a platinum/palladium covering. Observation was done using a JEOL MODEL 1200 scanning electron microscopy (SEM).

#### Separation of Hemocytes by Discontinuous Density Gradient Centrifugation

Hemolymph (0.5–1.5 mL) was withdrawn from the posterior adductor muscle of each animal using a 25-gauge needle, then collected and diluted 1:1 in a modified antiaggregant Alsever solution (MAS) (20.8 g/l glucose; 8 g/l Na citrate; 3.36 g/l EDTA; 22.5 g/l NaCl in distilled water). The hemocytes were then collected by centrifugation at  $\times 640$ g (4°C, 10 min). The commercial Percoll (Sigma) solution was adjusted to 1.100 mOsm by adding NaCl to a final concentration of 0.41% (w/v) and the gradients (10%, 30%, 50% and 70% (v/v)) were prepared in MAS. Oyster hemocyte pellets were resuspended in MAS and layered onto the top of a Percoll gradient composed of 70%, 50%, 30% and 10% Percoll. After centrifugation at  $\times 640$ g (4°C, 15 min), the hemocytes present at each density interface were collected separately with a syringe. The hemocytes appearing at interface 30/10% of the gradient were further separated by centrifugation through a Ficoll (type 400, Sigma) density gradient containing 20%, 15%, 10% and 5% (w/v) Ficoll prepared in MAS. After centrifugation, the cells appearing at each density interface were collected separately. The density gradient centrifugation was carried out in 13-mL tubes. Each gradient layer of 2.5 mL of Percoll or Ficoll solution and 2 mL of hemocyte suspension were layered in each tube. Each separated hemocyte subpopulations with Percoll gradient or Ficoll gradient were recovered by centrifugation and washed once in MAS. With Each separated hemocyte subpopulations at the different interfaces were made smears, and rapid hemacolor coloration was used.

#### Hemocyte Viability Evaluation

The viabilities of the fresh hemocytes collected from the oysters and the separated hemocyte subpopulations by Percoll discontinuous density gradient centrifugation were estimated by the 0.1% (w/v) Trypan blue test.

#### Phagocytosis of Zymosan

Zymosan (cell walls of *Saccharomyces cerevisiae*, zymosan A, Sigma) suspensions were prepared as described by Bachère et al. (1991). Zymosan particles at 40 mg 10 mL<sup>-1</sup> were suspended in sterile sea water (SSW) and boiled for 30 min, then washed twice and suspended in SSW before divided into aliquots and stored at -20°C. The aliquots were thawed and counted in a Mallassez cell immediately before use.

To study the phagocytosis of the external materials by oyster hemocytes, hemolymph from 20 oysters was extracted 1:3 in MAS and pooled. Eppendorf vials containing  $1 \times 10^6$  hemocytes (he) were prepared, then centrifuged ( $\times 200$  g, 10 min, 4°C) to remove MAS. One milliliter of filtered seawater (FSW) containing 2.5% of MAS was then added to the vials. Phagocytosis assays were carried out by adding zymosan (zy) suspensions to the Eppendorf vials. The ratios of zy/he were 5:1. The assays were carried out at room temperature (20–23°C) for 60 min. Smears were performed at the end of phagocytosis assay. Hemocytes were fixed and stained with Hemacolor kit. The percentage of cells with phagocytosed particles was evaluated in 30 random selected microscope fields at a magnification of  $\times 1,000$  in the slides.

To study the phagocytosis process with electron microscopy, hemolymph from five oysters was extracted in MAS (1:3) and pooled. Phagocytosis vials consisted of  $1 \times 10^6$  hemocytes, 5zy/he, and 80  $\mu$ L of MAS and FSW to a final volume of 2 mL. These assays were carried out at room temperature (20°C to 23°C) for 60 min. After this time, cells were fixed (1 h, 4°C) by adding 2.5% glutaraldehyde to the phagocytosis vials. Hemocytes were then washed for 2 h, 4°C in 0.1 M PIPES buffer containing 7% sucrose (pH = 7.2), postfixed in 1% osmium tetroxide and embedded in agar and epon. Ultrathin sections (50–70 nm) were contrasted with uranyl acetate and lead citrate.

#### Statistical Analysis

The SPSS software was used for statistical analysis. Differences in all studied parameters were evaluated by 1-way ANOVA followed by Tukey test for comparisons. LSD test was used for multiple comparisons. Values of  $P < 0.05$  were considered significant.

## RESULTS

#### Light Microscopy Observation

On Hemacolor smears, 2 hemocyte types were distinguished by light microscopy: granulocytes and hyalinocytes (or agranulocytes) according to the presence or the absence of cytoplasmic granules. Hyalinocytes appeared to have 2 types: small and large hyalinocyte cells, according to the cell diameter in size (Fig. 1). The granulocytes were abundant and often appeared as either spherical cells (round hemocytes) or amoebocytes (spreading hemocytes) on the smears. The granulocytes contained numerous basophilic or refringent cytoplasmic granules and had small nuclei, which often showed the oval or eccentric shapes. On smears, endoplasm and ectoplasm of the cytoplasm could be clearly distin-



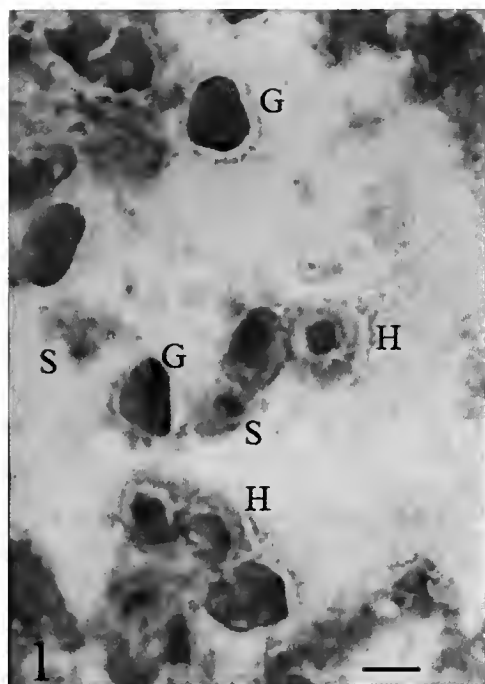


Figure 1. Total hemocyte population of *C. ariakensis* on smears stained with the Hemacolor kit (Merck). G, granulocyte; H, large hyalinocyte; S, small hyalinocyte. Bar, 6.0  $\mu\text{m}$ .

guished in the granulocytes. The ectoplasm presenting some thin pseudopodia showed a hyaline aspect whereas endoplasm was denser and contained cytoplasmic granules (Fig. 2). The large hyalinocytes were less abundant and often showed the round or oval shape. They often had the round and large nuclei in the center

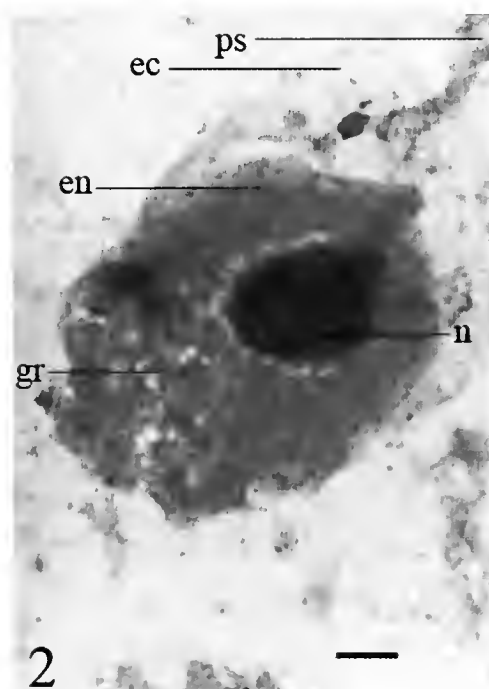


Figure 2. Light micrograph showing the granulocyte. en, endoplasm; ec, ectoplasm; ps, pseudopod; gr, cytoplasmic basophilic granules; n, nucleus. Bar, 1.5  $\mu\text{m}$ .

of the cells and reduced acidophilic cytoplasm, generally without granules or with few granules in the cytoplasm (Fig. 1). The small hyalinocytes were the least abundant on the smears. The cells and the nuclei often showed the round shape, and they showed large nuclei and reduced acidophilic and thin cytoplasm. Cytoplasmic granules were seldom observed in the small hyalinocytes (Fig. 1). In addition, multinucleate (2–4 nuclei) hemocytes with cytoplasmic granules were observed on these smears (Fig. 3). These multinucleate structures could be the result of the fusion of granulocytes.

#### Hemocyte Count and Measurements

Total hemocyte counts gave a mean ( $\pm$ SE) concentration of  $(2.06 \pm 0.20) \times 10^7$  cells  $\text{mL}^{-1}$  of hemolymph. A total of 1,016 hemocytes of eight oysters were counted for this study. Differential type hemocytes counts showed that the mean percentage compositions ( $\pm$ SE) of granulocytes, large hyalinocytes and small hyalinocytes were  $68.4 \pm 1.55$ ,  $21.6 \pm 1.21$  and  $9.0 \pm 0.74$  respectively. Table 1 showed the percentage of three cell types in the hemocyte population of *C. ariakensis*.

Table 2 showed the ranges and mean values ( $\pm$ SE) of the cell and nucleus sizes and the nucleus/cytoplasmic (N/C) ratios measured on Hemacolor stained smears. Granulocyte types showed larger sizes and smaller N/C ratios than hyalinocytes. Granulocytes were about  $6.8 \pm 0.15$   $\mu\text{m}$  and ranged from 3.2–9.5  $\mu\text{m}$ . Their nuclei were about  $2.08 \pm 0.05$   $\mu\text{m}$  and ranged from 1.29–4.23  $\mu\text{m}$ . The large hyalinocytes are homogenous in size but smaller than granulocytes, with about  $3.8 \pm 0.08$   $\mu\text{m}$  in diameter ranging from 2.35–6.35  $\mu\text{m}$ . Their nuclei were about  $2.20 \pm 0.05$   $\mu\text{m}$  and ranged from 1.2–3.07  $\mu\text{m}$ . The small hyalinocytes had an average size of  $2.05 \pm 0.04$   $\mu\text{m}$ , ranging from 1.32–2.78  $\mu\text{m}$ . Their nuclei diameter was  $1.20 \pm 0.03$   $\mu\text{m}$ , and ranged from 0.78–1.75  $\mu\text{m}$ . Their N/C ratios were the highest, but different among cells.

The results of the ANOVA comparison indicated a significant

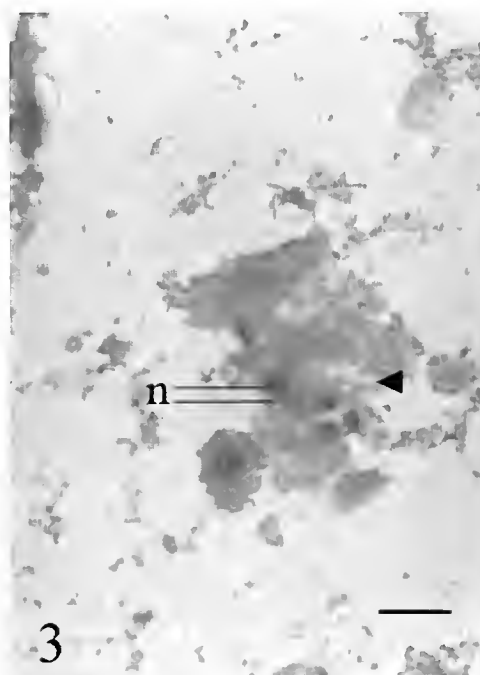


Figure 3. Light micrograph showing multinucleate hemocyte (arrow-head), n, nuclei. Bar, 15.0  $\mu\text{m}$ .

TABLE 1.

The percentage of 3 cell types (granulocytes, large hyalinocytes and small hyalinocytes) in the hemocyte population of *Crassostrea ariakensis* analyzed by the SPSS software.

Type of cell	Cell Mean <sup>a</sup> ± SEM	N	Sum (1016)	Percentage ± SEM
Granulocytes	86.7500 ± 4.45112	8	694	68.4125 ± 1.54796
Large Hyalinocytes	28.8750 ± 2.40117	8	231	22.5958 ± 1.20902
Small Hyalinocytes	11.375 ± 1.05115	8	91	8.9918 ± 0.73910

A total of 1,016 Haemocytes were distinguished and counted on Hemacolor's stained smears.

<sup>a</sup> Mean of cells on 8 smears. N, the observed smear size

difference for the cell size, N/C ratio and nucleus size. The multiple comparisons with the LSD indicated a significant difference between all hemocyte types for the cell size. However, in the case of the N/C ratio, multiple comparisons indicated no significant difference between the large and small hyalinocytes, and in the case of the nuclear diameter, multiple comparisons indicated no significant difference between the large hyalinocytes and granulocyte types (Table 2).

#### Electron Microscopy

Electron microscopy permitted us to confirm the occurrence of three hemocyte types: granulocytes, large and small hyalinocytes in the hemolymph of *C. ariakensis*. The large hyalinocytes often showed round or oval shapes, and presented smooth surface. The large hyalinocytes presented the high N/C ratios and thin cytoplasm, which contained a variable number of mitochondria, Golgi complex and endoplasmic reticulum (Fig. 4–6). They showed a total absence of cytoplasmic granules or a few small electron-lucid vesicles of different sizes, some of them probably originating in the Golgi complex or the smooth endoplasmic reticulum (Fig. 4). The nucleus of the hyalinocyte appeared round or oval and often was in a central position of the cell. Some large hyalinocyte nuclei had abundant euchromatin, and some showed abundant heterochromatin in the central and the peripheral positions. The nuclei of some hyalinocytes were surrounded only by small cytoplasmic rim (Fig. 5).

The small hyalinocytes were the most homogeneous in shape and showed round or oval. They had the highest N/C ratios and contained very thin cytoplasm. They showed a total absence of

cytoplasmic granules. The organelles such as Golgi complex and endoplasmic reticulum were not observed in the cytoplasm; however, one or two mitochondria were sometimes observed in the cytoplasm (Fig. 6, 7). The nucleus often appeared oval and held most position of the cell. The nucleus sometimes showed abundant heterochromatin in the central and the peripheral positions (Fig. 6).

The granulocytes showed more polymorphic than the hyalinocytes, and were oval or eccentric in shape. The most prominent features of the granulocytes were the numerous pseudopodia sprouting off their surfaces (Fig. 8, 9, 10), which were suggested related with the phagocytic ability of the granulocytes. Some pseudopodia of the granulocytes were slim and long, however some were thick and short (Fig. 8). Some pseudopodia compected together (Fig. 10). Engulfed vacuoles and residual bodies were also observed in the granulocytes. The granulocytes showed abundant cytoplasm and low N/C ratios. They presented similar organelles but on the contrary had abundant electron-dense particles or electron-lucent granules in the cell cytoplasm. The electron dense granules were spherical, often different in size and measured 0.2–0.4 µm in diameter. The electron-dense granules were composed of a homogenous electron-dense matrix (Fig. 8). The electron-lucent granules were round, with an electron-lucent core, surrounded by an electron-dense membrane unit and often very different in size and measured 0.2–0.6 µm in diameter (Fig. 9, 10). Some electron-lucent granules were rough along the peripheral; some were very smooth along the peripheral. Their nuclei appeared polymorphic such as round shape, kidney shape and bell shape (Figs. 8, 9). They often were in one end of the cells or in eccentric positions in the cell cytoplasm.

#### Scanning Electron Microscopy Observation

Observation under SEM confirmed the surface structures of the hemocytes. According to the size and the surface structure, 3 hemocyte types could be identified: (1) large cells that were usually round and showed no pseudopodia with relatively smooth surfaces, however, sometimes they showed some tiny refractive inclusions or spherule (Fig. 11); (2) small round cells that were the smallest, they were usually round and showed no pseudopodia with relatively smooth surfaces (Fig. 12); (3) irregular cells that often were irregular in shape and presented abundant tenuous and long pseudopodia they usually appeared irregular twist, poly-angle shapes and honeycomb-like surface structure; the surfaces of these cells were usually with corrugation, spongy projections and surface secretion particles (Fig. 13).

TABLE 2.

Mean values (µm) ± Standard Error and ranges of cell and nuclear diameters and nuclear/cytoplasmic (N/C) ratio of hemocytes of *Crassostrea ariakensis*.

Hemocyte type	N	Cell diameter ± sem	Nuclear diameter ± sem	N/C ratio ± sem
Granulocyte	107	6.7937 ± .14657 <sup>a</sup>	2.0816 ± .05408 <sup>a</sup>	.3144 ± .01061 <sup>a</sup>
Ranges		3.2857–9.5	1.29–4.23	0.171291–0.79520
Large Hyalinocytes	92	3.7637 ± .07966 <sup>b</sup>	2.1959 ± .04491 <sup>a</sup>	.5924 ± .01020 <sup>b</sup>
Ranges		2.35–6.35	1.2–3.07	0.321809–0.822148
Small Hyalinocytes	68	2.0490 ± .04052 <sup>c</sup>	1.2013 ± .03312 <sup>b</sup>	.5988 ± .01809 <sup>b</sup>
Ranges		1.32–2.78	0.78–1.75	0.337662–0.890052

Measurements were made on Hemacolor's stained smears. N, sample size.

The multiple comparisons were made by LSD. Different letters (a, b, c) at the same column showed significant difference, and the same letters (a, b, c) at the same column showed no significant difference between the mean values. Values of *P* < 0.05 were considered significant.

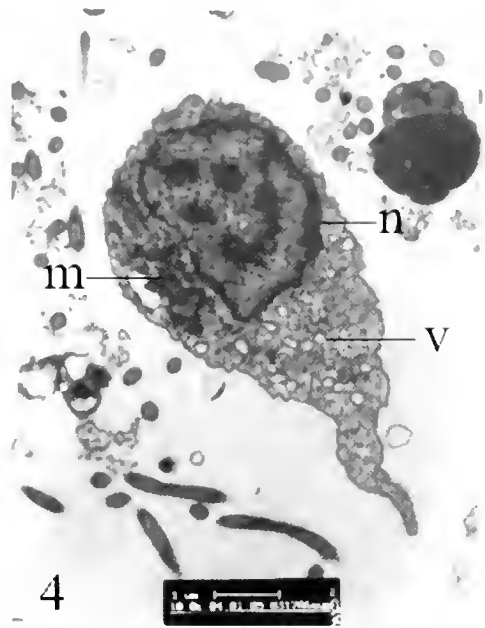


Figure 4. The large hyalinocyte presented an oval nucleus, a variable number of mitochondria and a few small electron-lucid vesicles. n, nucleus; m, mitochondrion; v, small electron-lucid vesicles. Bar, 1.0  $\mu$ m.

#### Separation of Hemocytes

After centrifugation in Percoll gradients, the total hemocyte population was separated into three cell fractions. Fraction present at the interface 10/30% contained only agranular hemocytes in-

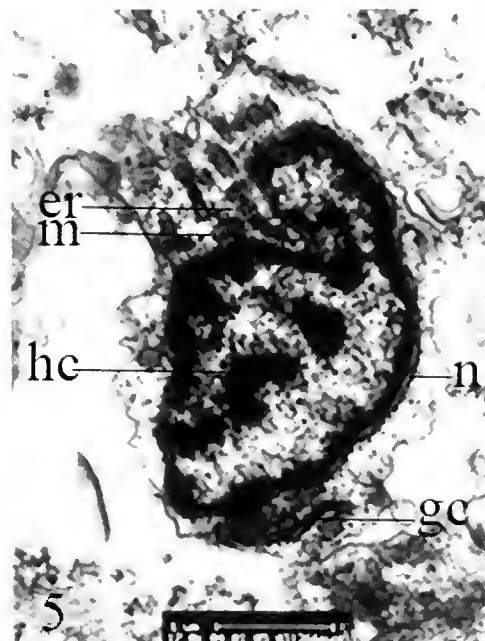


Figure 5. The large hyalinocyte presented an irregular nucleus containing abundant heterochromatin in the central and the peripheral positions. The large nucleus was surrounded by a small cytoplasmic rim. The large hyalinocyte showed a total absence of cytoplasmic granules. However, mitochondria, Golgi complex and endoplasmic reticulum were observed in the cytoplasm. er, endoplasmic reticulum; gc, Golgi complex; hc, heterochromatin; m, mitochondrion; n, nucleus. Bar, 1.0  $\mu$ m.

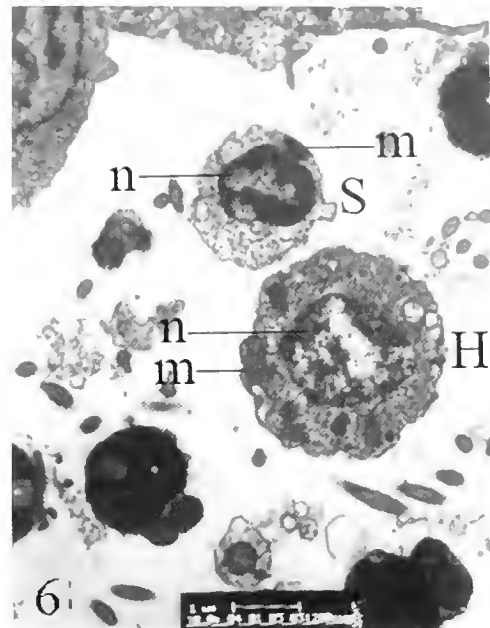


Figure 6. The large hyalinocyte and the small hyalinocyte were both round, and their nuclei were also round. Several mitochondria were observed in the cytoplasm of the large hyalinocyte and only one mitochondrion was found in the cytoplasm of the small hyalinocyte. H, large hyalinocyte; S, small hyalinocyte; m, mitochondrion; n, nucleus. Bar, 1.0  $\mu$ m.

cluding the large and small hyalinocytes. Fraction collected from the interface 50/70% was composed of pure granulocytes. Fraction appearing at the interface 30/50% was a mixture of all hemocyte types.

However, the large and small hyalinocytes could not be separated with Percoll gradients or Ficoll gradient. By using Ficoll

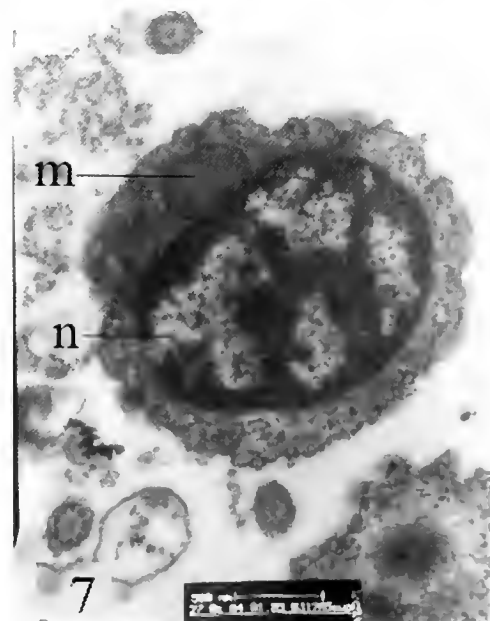


Figure 7. Higher magnification of the small hyalinocyte showing a total absence of cytoplasmic granules and thin cytoplasm. Only one mitochondrion was found in the cytoplasm. m, mitochondrion; n, nucleus. Bar, 0.5  $\mu$ m.

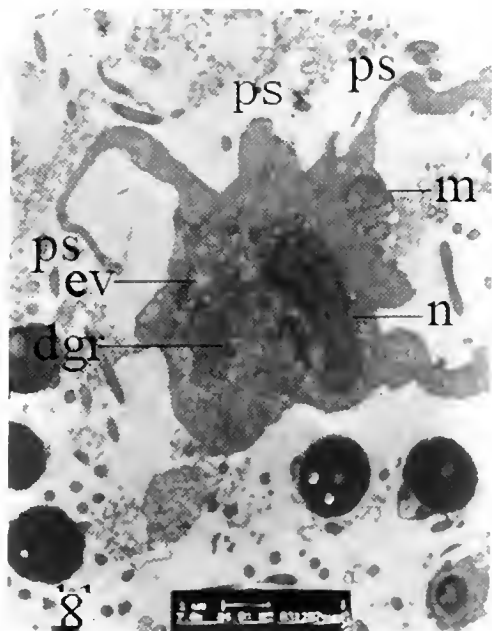


Figure 8. The granulocyte showed abundant cytoplasm and low nucleus: cytoplasm ratio. The most prominent feature of the granulocyte was the pseudopodia (ps) sprouting off its surface. Some pseudopodia of the granulocytes were slim and long, however some were thick and short. Abundant electron-dense granules (dgr) and bell-shaped nucleus (n) in the cell cytoplasm were also observed. ev, engulfed vacuole; m, mitochondrion; Bar, 1.0  $\mu$ m.

gradient, both fractions at the interface of 10/15% and 5/10% consisted of the large and small hyalinocytes simultaneously; the fraction at the interface of 15/20% contained cells whose composition was variable in different experiments.

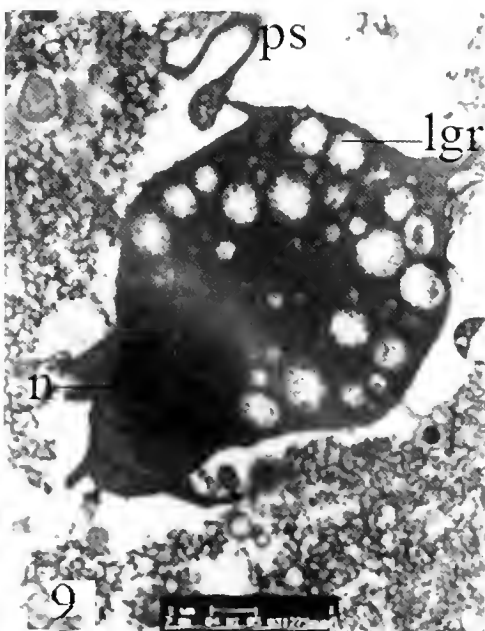


Figure 9. The granulocyte presented a round nucleus (n), abundant cytoplasm and electron-lucent granules (lgr) in the cytoplasm. The pseudopodia (ps) sprouting off its surface. Bar, 1.0  $\mu$ m.

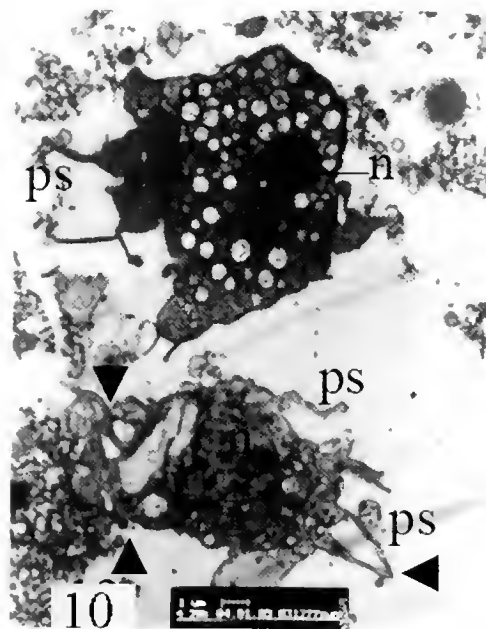


Figure 10. Many slim pseudopodia (ps) sprouting off the granulocyte surface, some pseudopodia completed together (arrowhead). n, nucleus. Bar, 1.0  $\mu$ m.

#### *Hemocyte Viability After Separation*

The dead cells appeared blue staining with the Trypan blue, and the live cells did not stain with the Trypan blue (Fig. 14). The viability of the separated hemocytes collected from fractions at the interface 10/30% and 30/50% of Percoll gradients were compared with the nonseparated hemocytes. For the total hemocyte popula-

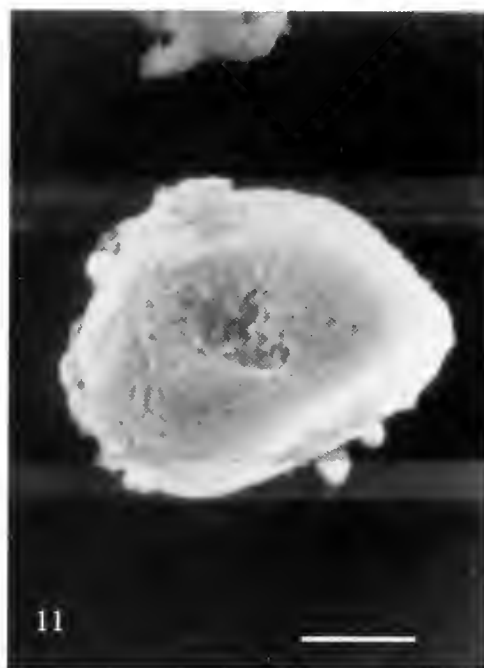


Figure 11. Scanning electron micrograph showed the large round cell was usually round and presented a relatively smooth surface, sometimes presenting some tiny refractive inclusions or spherule. Bar, 1.3  $\mu$ m.



Figure 12. Scanning electron micrograph showed the small round cell was the smallest, usually round, having a smooth surface. Bar, 0.6  $\mu\text{m}$ .

tion before separation, the mean cell viability was 86.4%. After separation, the corresponding values for fractions at the interface 10/30% and 30/50% of Percoll gradients were 81.8, 82.8%, respectively. No significant difference (*t*-test) was detected between



Figure 13. Scanning electron micrograph showed the irregular cell appeared twist in shape or irregular poly-angle shapes and honeycomb-like surface structure. The surface of the cells was usually with corrugation, spongy projections, and surface secretion particles. The surface of the cell had abundant tenuous and long pseudopodia. Bar, 2.1  $\mu\text{m}$ .

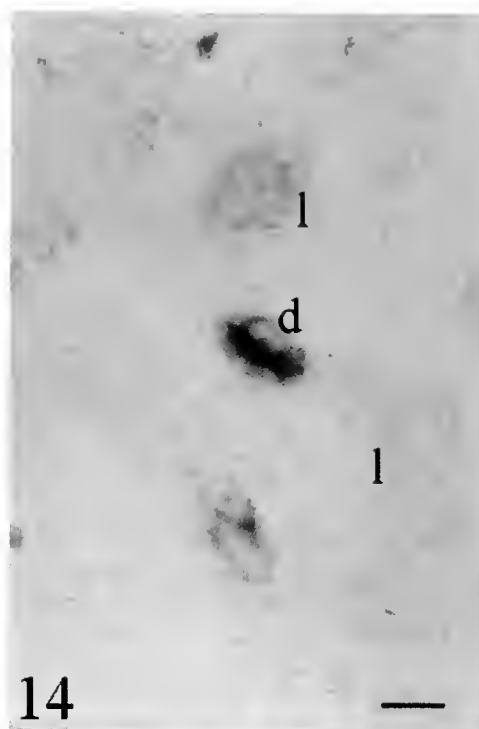


Figure 14. Trypan blue test. The dead cells (d) appeared blue staining with the Trypan blue, and the living (l) did not stain with the Trypan blue. Bar, 6.0  $\mu\text{m}$ .

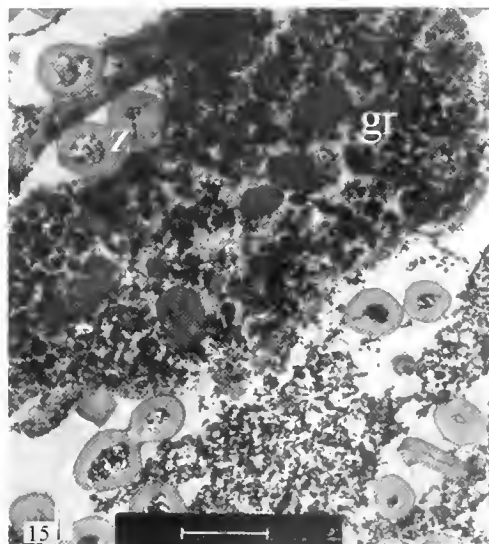
the viabilities of the total hemocyte population before separation and any of the two separated cell fractions.

#### Phagocytosis

*In vitro* phagocytosis assays showed that among the hemocyte types, the granulocytes showed an important phagocytic activity whereas the hyalinocytes did not. Light microscopy revealed that several zymosans were located inside the hemocytes after 60 min, and a few particles were encircled by pseudopodia. The number of zymosan particles phagocytosed per hemocyte was variable, and  $6.24 \pm 0.74\%$ ,  $20.61 \pm 1.19\%$  and  $29.07 \pm 1.08\%$  of hemocytes phagocytosed, one, two or three particles, respectively, whereas  $39.08 \pm 1.69\%$  of hemocytes phagocytosed four or more particles. Electron microscopy confirmed the intracellular location of zymosan. Particles were found inside phagosomes (Fig. 15), some of the particles partially degraded. Hemocytes containing zymosan had many vacuoles that included degraded products.

#### DISCUSSION

Morphological criteria were generally used to characterize hemocytes in bivalves, however, the existing nomenclature of bivalve hemocytes is inconsistent, being dependent on the observer and the technique used (Feng et al. 1971, Ruddell 1971a, Ruddell 1971b, Foley & Cheng 1972, Cheng 1975, Cheng 1981, Moore & Lowe 1977, Hawkins & Howse 1982, Rasmussen et al. 1985, Chang et al. 2005). Cheng (1981) presented a morphological scheme based on numbers of cytoplasmic granules, dividing cells into 2 types: granulocytes, cells containing granules that ranged from very few to numerous; and agranulocytes, cells containing few or no granules. In this study, we identified 2 main hemocyte types in *C. ariakensis*, with both light and electron microscopy



**Figure 15.** Transmission electron micrograph showed Zymosan particles phagocytosed by *Crassostrea ariakensis* haemocyte. Z, zymosan; gr, granule. Bar, 1.5  $\mu$ m.

observation: granulocytes and agranulocytes (large hyalinocytes and small hyalinocytes). The granulocytes were characterized by the abundant content of granules, presenting a noncentral small nucleus, spreading with pseudopodia related with the phagocytic abilities. According to the cell size, agranulocytes appeared to have two types: small hyalinocytes and large hyalinocytes. The large hyalinocytes were morphologically characterized by the relatively large and central nuclei surrounded by the small volume of cytoplasm and by few or no cytoplasmic granules. The small hyalinocytes have the least cell diameter and large and central nuclei surrounded by the small volume of cytoplasm with the absence of cytoplasmic granules. Many authors suggested that the hyalinocytes were non-different cells; however, hyalinocytes were classed into 2 types, small and large hyalinocytes, by Xue et al. (2000), which supported our classification. Some studies suggested that it was possible to distinguish acidophilic and basophilic granulocytes according to the staining affinities of the cytoplasmic granules of the granulocytes (Cheng 1975, Cheng 1981, Suresh & Mohandas 1990, Nakayama et al. 1997, López et al. 1997b, Wootton & Pipe 2003, Zhang et al. 2005). However, Xue et al. (2000) found the granulocytes of *Ostrea edulis* contained only numerous basophilic or refringent cytoplasmic granules, in agreement with our study on the staining characteristic of *C. ariakensis* granulocytes.

In addition, the multinucleate granulocytes were observed on smears. These cells have been observed in other mollusks (Sparks & Pauley 1964, Cheng 1981, Anderson 1987, Wootton & Pipe 2003) with light microscopy. They are considered to be the result of a fusion of granulocytes in some pathological conditions such as postmortem changes or rejection of grafts. However, the origin and progress of their forming are not clear.

The ultrastructure of the hemocytes in this study revealed that granulocytes mainly contained two types of granules: electron-dense particles and electron-lucent granules. They were different in size of both types of granules. Furthermore, the granules of different size often existed inside most granulocytes simultaneously. Therefore, granulocytes were not differentiated in this study. Some investigators distinguished the granulocytes in other

bivalve with only small or large granules and classed the granulocytes into large and small granulocytes. They considered that the granulocytes with small or large granules were immature or mature granulocytes, respectively (Klebanoff & Clark 1978, Rasmussen et al. 1985, Pipe 1990). However, we agree with the option of Zhang et al. (2005) that the peculiarity of granules should be determined by their origin or function, not by their size and did not believe that granulocytes with different type of granules were in different development phases, though their origin and functions were not wholly clear.

The ultrastructural study of *C. ariakensis* hemocytes showed that some large hyalinocytes contained nuclei with abundant euchromatin and others contained nuclei with large clumps of heterochromatin. Carballal et al. (1997c) also found this phenomenon, but they suggested that both types of hyalinocytes could belong to a different cell line, one for hyalinocytes and another for granulocytes, or hyalinocytes containing abundant euchromatin might give rise to hyalinocytes with more heterochromatin and granulocytes. However, we consider that maybe the both types of large hyalinocytes are in different development phases of the hemocytes, the large hyalinocytes containing nuclei with abundant euchromatin can give rise to hyalinocytes with more heterochromatin that are overripe. The most granulocytes containing nuclei with abundant euchromatin probably are in their bloom stage and present more phagocytic competence. Maybe some large hyalinocytes containing nuclei with abundant euchromatin also result from the overripe granulocytes after fulfilling their phagocytic functions because the nuclear diameters were not significantly different between the large hyalinocytes and the granulocytes.

There are several theories on bivalve hemocytes renewal and maturation. Moore and Eble (1977) suggested that different hemocytes are maturing stages within a single cell line. Cheng (1981) proposed an ontogenetic model with two cell lines, one for hyalinocytes and another for granulocytes, each originating from a different prohemocyte. Auffret (1988) also suggested this last hypothesis for *O. edulis* and *C. gigas* hemocytes. However, in our study the morphological variability found by light and electron microscopy in the oyster hemocytes do not allow us to confirm any of the previous hypotheses.

Total hemocyte counts showed high variability in the number of circulation hemocytes because the density of hemolymph might vary with different species, age and physical status. Because spreading ability was different between hyalinocytes and granulocytes, results of cell measurements were different according to the method used. Lopez et al. (1997a, 1997b) suggested that fixing hemocytes in suspension before measuring was a better method. In our study, differential hemocyte counts after fixing identified granulocytes as the predominant cell type (68.4%), followed by the large hyalinocytes (21.6%) and the small hyalinocytes (9.0%). In the study of other mollusks, similar results were reported. The granulocytes corresponded to roughly 75% and 66% of the total population of hemocytes and the hyalinocytes corresponded to the remaining 25% and 34% in *Scrobicularia plana* (Wootton & Pipe 2003) and *Argopecten irradians* (Xing et al. 2002) respectively. However, some authors reported the hyalinocytes were the predominant cell type (i.e., granulocytes/hyalinocytes were 44.7%/55.3% and 37.3%/62.7%) in *A. irradians* (Zhang et al. 2005) and *C. virginica* (Hégaret et al. 2003) respectively.

The significant differences of cell size and N/C ratios existed in either hyalinocytes or granulocytes. Granulocyte types showed larger sizes and smaller N/C ratios than hyalinocytes. These fun-



damental features are common to many bivalve species (e.g., *M. mercenaria*; Foley & Cheng 1974, *C. virginica*; Feng 1965, *C. edulis*; Russell-Pinto et al. 1994, *M. edulis*; Rasmussen et al. 1985, Friebe & Renwranz 1995, *M. lusoria*; Wen et al. 1994 and *A. irradians*; Zhang et al. 2005).

In SEM, special emphasis had been placed on the surface structure. The observation under SEM confirmed the pseudopodia observed under TEM. Moreover, the irregular cells, large round cells and small round cells under SEM might correspond to the granulocytes, large hyalinocytes and small hyalinocytes observed under TEM.

The quantitative study of phagocytosis showed that there were functional differences between hemocyte types of *C. ariakensis*. The granulocytes were phagocytic cells. On the contrary, the hyalinocytes showed a limited phagocytic ability. Similar results were reported in *M. edulis* (Moore & Lowe 1977), *Tapes semidecussatus* (Montes et al. 1995), *Tridacna crocea* (Nakayama et al. 1997), *C. virginica* (Foley & Cheng 1975, Renwranz et al. 1979), *M. mercenaria* (Foley & Cheng 1975), *Mytilus californianus* (Bayne et al. 1979), *O. edulis* and *C. gigas* (Mourton et al. 1992), *Cerastoderma edule* (Russell-Pinto et al. 1994), *Tiostrea chilensis* (Hine & Wesney 1994), *M. galloprovincialis* (Carballal et al. 1997c) and *A. irradians* (Zhang et al. 2005), but these reports were in contrast with those previously reported by Tripp (1992) on *M. mercenaria*, Lopez et al. (1997b) on *R. decussatus* and Cima et al. (2000) on *Tapes philippinarum*. In the latter species, the authors considered that the agranular hemocytes and granulocytes were both active phagocytes.

Phagocytic hemocytes require considerable amounts of energy in the process from phagocytosis of particles, secretion of hydrolysis enzymes, fusion and decomposition of granules to discharge of wastes. Therefore, phagocytic hemocytes often contained abundant numerous mitochondria, Golgi complex, endoplasmic reticulum, and a great quantity of glycogen. Moreover, abundant granules and many spreading pseudopodia of the phagocytic hemocytes were related to the phagocytic competence.

The role of bivalve hyalinocytes with no phagocytosis is unknown. It is possible that they develop other functions different from phagocytosis because in *Cerastoderma edule* they are involved in rosette formation with sheep erythrocytes (Russell-Pinto et al. 1994) and in other invertebrates, such as the crustaceans, they participate in the coagulation process (Hose et al. 1990).

Density gradient centrifugation is one of the most used techniques for separating molluscan hemocytes into subpopulations.

Different materials have been applied as gradient substrates. Cheng et al. (1980) separated fixed hemocytes of *C. virginica* into several subpopulations by sucrose density gradient centrifugation. Clearly, sucrose is not suitable for separation of live cells, in particular, when the separation aims at studies about cell function, because sucrose solutions cannot keep the same osmolarity at different concentrations. However, Percoll, a colloidal suspension of polyvinylpyrrolidone coated silica particles, has been extensively used for separation of living cells. Some investigators have applied it in separating hemocytes of *C. gigas* (Bachère et al. 1988), *C. virginica* (Cheng & Downs 1988), *Lymnaea stagnalis* (Adema et al. 1994), *M. edulis* (Friebe & Renwranz 1995) and *R. decussatus* (Lopez et al. 1997b) and *O. edulis* (Xue et al. 2000). The results obtained in this study indicated that it was also applicable to the separation of *C. ariakensis* hemocytes. After the separation in this type of density gradient, the granulocytes were separated from agranulocytes and pure granulocytes were obtained. However, the separation of different agranular cell types was not achieved by Percoll gradient centrifugation. The agranulocytes had the low percentage of population of hemocytes and were difficult in adhering on the smears, so the observed agranulocytes were fewer than the granulocytes after Percoll gradient centrifugation. In addition, the granulocytes were very stained because the Percoll was not wholly washed off.

Ficoll has very high viscosity, and Ficoll density gradient had been used to separate the large and small hyalinocytes of the *O. edulis* (Xue et al. 2000). In this study, we also applied the technique of centrifugal elutriation, however, the large hyalinocytes and small hyalinocytes could not be separated in this study. In *O. edulis*, the large hyalinocytes could be separated from the small hyalinocytes, but the separation was not complete. However, the density gradient centrifugation with successive Percoll and Ficoll solutions is a practical technique for molluscan hemocyte separation, although it has different efficiency with the different molluscan hemocytes.

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## ADVANCED *PERKINSUS MARINUS* INFECTIONS IN *CRASSOSTREA ARIAKENSIS* MAINTAINED UNDER LABORATORY CONDITIONS

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**ABSTRACT** The Suminoe oyster, *Crassostrea ariakensis*, has been under investigation since the early 1990s for potential use in restoring the commercial harvest or for aquaculture of oysters in the Chesapeake Bay, USA. Initial studies focusing on *C. ariakensis* documented a significant level of tolerance to the protozoan parasite *Perkinsus marinus*, a pathogen found in almost all reaches of the Bay and widely acknowledged as one of the main reasons for the decline in the eastern oyster, *Crassostrea virginica*, harvest since the late 1980s. *Crassostrea ariakensis* was demonstrated to acquire *P. marinus*, however infection intensities, as measured using Ray's thioglycollate medium assay indices, generally were found to be light. As part of a series of experiments to study potential impacts on the Chesapeake Bay region of pathogens found in *C. ariakensis* in Asia, a challenge experiment was conducted to study the pathogenicity of *Perkinsus olseni* to *C. ariakensis*. During this study, we observed the acquisition of moderate and heavy infection intensities of *P. marinus* in triploid *C. ariakensis* oysters being maintained in the laboratory. Results suggest that there may be some risk of mortality from *P. marinus* if *C. ariakensis* is held under stressful conditions at least in hatchery or laboratory settings.

**KEY WORDS:** *Perkinsus marinus*, *Crassostrea ariakensis*, PCR, RFTM, oyster

### INTRODUCTION

The eastern oyster, *Crassostrea virginica* (Gmelin, 1791), an integral part of the economy and ecology of the Chesapeake Bay, has been in a severe state of decline in recent decades. Two pathogens, *Haplosporidium nelsoni* (Haskin, Stauber & Mackin, 1966) and *Perkinsus marinus* (Mackin, Owen & Collier, 1950), the parasites responsible for the diseases known as MSX and dermo respectively, have contributed significantly to the decimation of the oyster populations in Chesapeake Bay since the 1950s (Sindermann 1990).

The decline in oyster production in Virginia led to the formation of a number of panels in the early 1990s to make recommendations on restoring oyster populations. One option currently being seriously considered is the introduction and use of a nonnative oyster in Chesapeake Bay. A Virginia Institute of Marine Science (VIMS) study conducted in 1996 focusing on the Pacific oyster, *Crassostrea gigas* (Thunberg, 1793), documented lower disease susceptibility in *C. gigas* than in the native eastern oyster, *C. virginica*; however, growth rates of the Pacific oyster were equal to or inferior to the native oyster in the Chesapeake Bay (Calvo et al. 1999). A 1998 field-based study on another Asian oyster, the Suminoe oyster, *Crassostrea ariakensis* (Fujita, 1913), documented rapid growth and survival in that species, as compared with *C. virginica*, even when endemic diseases were prevalent (Calvo et al. 2001). Although initial baseline samples of *C. ariakensis* in that study revealed a 12% prevalence of *P. marinus*, all subsequent samples collected after field deployment showed *C. virginica* with higher *P. marinus* prevalence than *C. ariakensis*. Additionally, Ray fluid thioglycollate medium (RFTM) (Ray 1952) diagnoses showed several heavy *P. marinus* infections in *C. virginica*, however, only light infections were observed in *C. ariakensis* (Calvo et al. 2001).

In this report we describe the first account of moderate to heavy *P. marinus* infections in *C. ariakensis*. The oysters in this study were held in laboratory aquaria at VIMS during a challenge experiment conducted to examine the potential pathogenicity of *Perkinsus olseni* Lester and Davis, 1981, to *C. ariakensis*, because *P.*

*olseni* was found during a recent survey of *C. ariakensis* populations in Asia (Moss & Reece 2005). Prior to the start of the challenge experiment, *C. ariakensis* were initially determined to be *Perkinsus* sp.-free, based on molecular diagnostics using PCR (Casas et al. 2002). The oysters acquired detectable levels of the parasite, however, after being held for about 8 weeks in the laboratory aquaria. We used the infected oysters in our challenge study, despite baseline *P. marinus* infection prevalence, to examine the potential for *C. ariakensis* oysters to become coinfecting with multiple *Perkinsus* species. We report here our observations regarding the *P. marinus* infection levels observed in *C. ariakensis* during this challenge experiment.

### MATERIALS AND METHODS

#### Experimental Design

On January 24, 2005, 120 market sized (~75 mm shell height) triploid *C. ariakensis* were received from the Aquaculture Genetics and Breeding Technology Center hatchery at VIMS. The animals were held for 4 days inside a plastic mesh bag in a holding tank prior to bringing them into the laboratory aquaria. The holding tank was not covered and there was flow-through of nonfiltered York River water of approximately 9°C and 16 ppt salinity. On removal from the holding tank, 20 *C. ariakensis* were immediately sacrificed, and gill and mantle tissues were excised aseptically from each animal for DNA extraction. Genomic DNA of each oyster was used in a PCR-based molecular diagnostic assay (Casas et al. 2002) to examine the animals for the presence of DNA from *Perkinsus* spp. parasites. The animals were subsequently held for 59 days in 10-gallon glass aquaria that were maintained at 20°C and 25 ppt salinity at a density of approximately 25 oysters per tank and were fed 0.1 g oyster<sup>-1</sup> algal food daily (Reed Mariculture, San Jose, CA). At the end of 59 days, on March 24, 2005, five *C. ariakensis* were sacrificed and gill and mantle were excised aseptically from each animal to be used in *Perkinsus* spp. screening by PCR assays as described later.

On March 28, 2005, the remaining *C. ariakensis* ( $n = 60$ ) were used in a challenge experiment designed to evaluate the pathogenicity of *P. olseni* to *C. ariakensis*. Twenty *C. ariakensis* received single pallial cavity injections of 100 µL of 25 ppt sterile artificial

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seawater (SASW) through a notch in the shell, and 40 animals were inoculated with  $10^5$  log phase cultured *P. olsenii* cells per gram body weight in a 100- $\mu$ L volume. The clonal *P. olsenii* culture isolated from a Japanese *Venerupis philippinarum* (Adams and Reeve, 1850) clam was obtained from Mr. Chris Dungan, Maryland DNR Cooperative Oxford Laboratory, Oxford Maryland, (ATCC PRA-181). Thirty additional untreated, control *C. ariakensis* were neither notched nor inoculated, but held in separate aquaria and otherwise maintained and fed in the same manner as the experimental animals for the duration of the experiment. Those *C. ariakensis* inoculated with 25 ppt SASW were maintained in one 10-gallon aquarium, and those inoculated with *P. olsenii* were split evenly between two aquaria. All aquaria were identical and each tank was covered with a plexiglass lid.

All aquaria environments were maintained at 20°C to 22°C and contained 25 ppt aerated, 1- $\mu$ m filtered York River water, two thirds of which was removed each week and replaced with an equal volume of clean, 25 ppt, 1- $\mu$ m filtered York River water. Animals were fed daily a single dose of approximately 0.1 g oyster<sup>-1</sup> algal feed (Reed Mariculture).

Aquaria were checked daily for oyster mortalities, and moribund animals were removed. The cumulative mortality was calculated for each sample as the sum of the mortalities that occurred during the course of the experiment, which started 59 days after the *C. ariakensis* were brought into the aquaria, divided by the number of live animals at the start of the experiment. When moribund animals were discovered, if adequate undegraded tissues remained they were processed for analysis. Gill and mantle tissues were removed with a portion preserved in 95% ethanol for DNA extraction and molecular diagnostics and gill, mantle and rectal tissues were processed for Ray fluid thioglycollate medium (RFTM) assay (Ray 1952). After 27°C incubation for 5–6 days in RFTM, tissues were removed from the culture tubes, macerated on microscope slides and stained with Lugol iodine. Stained, cover-slipped tissue preparations were examined under a light microscope and *Perkinsus* sp. tissue burdens were enumerated on a scale from rare (R) to very heavy (VH) based on the categories of Ray (1952, 1954). Visceral mass tissue sections were preserved in Davidson's solution for histological analysis (Shaw & Battle 1957).

### Experimental Sampling

Because of space constraints in the laboratory, the untreated *C. ariakensis* that were remaining after 37 days were sacrificed and tissues were taken for DNA, RFTM and histological analysis of disease status as described earlier. For those tanks in which *C. ariakensis* were inoculated with either 25 ppt SASW (one tank) or with *P. olsenii* (two tanks), two randomly chosen oysters from each tank were removed and sacrificed for disease diagnosis on days 21, 44 and 59 postinoculation. The challenge experiment was terminated on day 72 and all remaining animals were sacrificed and tissues preserved as mentioned earlier for DNA, RFTM and histological analysis.

### Nucleic Acid Extraction

Genomic DNA was extracted from the excised mantle and gill tissue snips, using a DNeasy Tissue Kit (Qiagen Inc., Valencia, CA), following manufacturer's protocols, except that DNA was eluted in a single 200- $\mu$ L volume of elution buffer after a 10 min incubation at room temperature (approximately 20°C).

### SSU Genes

To assure that PCR amplifiable DNA was present in all extracted samples, genomic DNAs were tested using universal small subunit (SSU) ribosomal RNA gene primers 16S-A (5' CCG AAT TCG TCG ACA ACC TGG TTG ATC CTG CCA GT 3') and 16S-B (5' GGA TCC AAG CTT GAT CCT TCT GCA GGT TCA CCT AC 3') (modified from Medlin et al. 1988) with an expected amplification product of approximately 1,800 bp. Each 25- $\mu$ L reaction contained 20 mM Tris-HCl (pH8.4), 50 mM KCl, 0.75 mM MgCl<sub>2</sub>, 0.1 mM of each dNTP, 0.5  $\mu$ M each primer, 0.0125 U  $\mu$ L<sup>-1</sup> *Taq* polymerase, 0.2 mg mL<sup>-1</sup> BSA and 0.5  $\mu$ L genomic DNA (~10–50 ng total). Amplifications were performed with an initial denaturation of 94°C for 4 min, followed by 35 cycles at 94°C for 30 sec, 45°C for 30 sec, 65°C for 2 min, with a final elongation of 65°C for 2 min. After amplification, 3  $\mu$ L of PCR product were analyzed by agarose gel electrophoresis (2%), stained with ethidium bromide and visualized under UV light. Images were recorded with an Alpha Innotech FlourChem (San Leandro, CA) imaging system.

### Genus-specific *Perkinsus* sp. PCR Assay

Screening for *Perkinsus* sp. DNA was performed using *Perkinsus* genus-specific primers, PerkITS-85 (5' CCG CTT TGT TTG GAT CCC 3') and PerkITS-750 (5' ACA TCA GGC CTC TAA TGA TG 3') (Casas et al. 2002) that target the internal transcribed spacer (ITS) region of the ribosomal RNA gene complex. Each PCR reaction contained the following: PCR buffer at a concentration of 20 mM Tris-HCl (pH8.4), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP each primer at 0.1  $\mu$ M, 0.025 U  $\mu$ L<sup>-1</sup> *Taq* polymerase, 0.05 mg mL<sup>-1</sup> BSA and 0.5  $\mu$ L genomic DNA (~10–50 ng total). Amplifications were performed with an initial denaturation of 95°C for 4 min followed by 40 cycles of 95°C for 1 min, 53°C for 1 min, 65°C for 3 min, with a final elongation of 65°C for 5 min. After amplification, 4  $\mu$ L of PCR product were analyzed as described earlier.

### *Perkinsus* Species-specific Assays

Identification of the *Perkinsus* species that was infecting animals shown to have positive amplification products with the *Perkinsus* genus-specific assay was accomplished through the use of *P. marinus* and *P. olsenii* species-specific PCR assays. *Perkinsus marinus*-specific primers PmarITS-70F (5' CCT TTG YTW GAG WGT TGC CAG ATG 3') and PmarITS-600R (5' CGA GTT TGC GAG TAC CTC KAG AG 3') (Audemard et al. 2004) and *P. olsenii*-specific primers designed for this study Pols-140F (5' GAC CGC CTT AAC GGG CCG TGT T 3') and PolsITS-600R (5' GGR CTT GCG AGC ATC CAA AG 3') were used in separate 25- $\mu$ L reactions. PCR reactions for the *P. marinus* ITS region contained the following: PCR buffer at a concentration of 20 mM Tris-HCl (pH8.4), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM each dNTP, each primer at 0.1  $\mu$ M, 0.025 U  $\mu$ L<sup>-1</sup> *Taq* polymerase, 0.05 mg mL<sup>-1</sup> BSA and 0.5  $\mu$ L genomic DNA (~10–50 ng). Amplifications were performed with an initial denaturation of 95°C for 4 min followed by 40 cycles of 94°C for 1 min, 57°C for 1 min, 65°C for 3 min, with a final elongation of 65°C for 10 min. PCR reactions for the *P. olsenii* ITS region contained the following: PCR buffer at a concentration of 20 mM Tris-HCl (pH8.4), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM each dNTP, each primer at 0.1  $\mu$ M, 0.025 U  $\mu$ L<sup>-1</sup> *Taq* polymerase, 0.05 mg mL<sup>-1</sup> BSA and 0.5  $\mu$ L genomic DNA (~10–50 ng). Thermocycling parameters were as follows: an

initial denaturation of 95 °C for 4 min followed by 40 cycles of: 94 °C for 1 min, 62 °C for 1 min, 65 °C for 3 min, all followed by a final elongation step of 65 °C for 10 min. After amplification, for each species-specific reaction, 4 µL of PCR product were analyzed as described earlier.

Specificity of *P. olseni* primers was tested against *P. marinus* and *Perkinsus chesapeaki* McLaughlin, Tall, Shaheen, Elsayed and Faisal 2000, DNAs. In addition, amplification products from positive *P. olseni*-specific reactions were sequenced. PCR products were cloned into the plasmid pCR4-TOPO and transformed into *E. coli* using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) following the manufacturer's protocols. Transformed bacterial colonies were picked from agar plates using a sterile wooden toothpick and were inoculated into 10 µL of sterile water in 200 µL plastic strip tubes. Inoculated water samples were boiled for 4 min at 94 °C and 0.5 µL of the boiled preparation was used in an M13 PCR reaction using the forward and reverse primers supplied in the cloning kit. Each 25 µL reaction contained the following: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM each dNTP each primer at 1 µM, 0.25 U µL<sup>-1</sup> *Taq* polymerase, and 0.2 mg mL<sup>-1</sup> BSA. Thermocycling parameters were as follows: an initial denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 sec, 54 °C for 30 sec and 72 °C for 1 min, followed by a final elongation at 72 °C for 5 min. Following amplification using the M13 primer pairs, 3 µL of each PCR product were analyzed as mentioned earlier. Prior to sequencing, PCR products from clones containing the correct insert size were treated with shrimp alkaline phosphatase (SAP) and exonuclease I (*Exo* I) (Amersham Biosciences, Piscataway, NJ), to degrade nucleotides and single-stranded DNA (primers) remaining after PCR. Five micro liters of the M13 PCR product were combined with 0.5 units of SAP and 5.0 units of *Exo* I, and incubated at 37 °C for 30 min, 80 °C for 15 min, and 15 °C for at least 5 s. Clean PCR products from plasmid inserts were sequenced bidirectionally using the Big Dye Terminator kit (Applied Biosystems, Norwalk, CT) with M13 sequencing primers, and using 5-µL reactions with 0.125 times the concentration of Big Dye reagent specified in the manufacturer's protocols. Each 5 µL reaction contained 0.0625 µL of Big Dye, 0.96875 µL of 5x buffer, 1.6 pmol of each primer, and 10 ng of clean PCR product. Thermocycling parameters were as follow: 25 cycles of 96 °C for 1 min, 96 °C for 10 sec, 50 °C for 5 sec, 60 °C for 4 min, followed by a final incubation at 4 °C until the sequencing reaction products were precipitated using the ethanol/sodium acetate precipitation method (ABI User Bulletin, April 11, 2002). Precipitated sequencing reaction products were resuspended in 20 µL of Hi-Di formamide (Applied Biosystems) and 10 µL of each were electrophoretically separated on an ABI 3100 Prism Genetic Analyzer.

#### RFLP

For further confirmation of the presence of *P. marinus* or *P. olseni* DNA, and to further verify the specificity of the *Perkinsus* species-specific primer sets, genus-specific PerkITS-85/750 amplification products were digested with *Mbo* I restriction endonuclease (New England Biolabs, Beverly, MA). Based on analyzing published and unpublished sequence data of all known *Perkinsus* species, this enzyme will cut the approximately 760 bp ITS fragment of *P. marinus* DNA that is generated in the genus-specific assay to yield a 3-fragment digestion profile unique to this species. The fragments should be approximately 17,226 and 264 bp in length. For *P. olseni*, *Mbo* I should cut the ITS region fragment to

yield a 4-fragment digestion profile with fragment lengths of approximately 64, 85, 251 and 262 bp in length. Each 10-µL digestion reaction contained 5 µL of PCR product, 8.2 µL of sterile distilled water, 1.5 µL of 10X NEB buffer 3, and 0.3 µL of restriction enzyme. As controls, separate oyster genomic DNA samples, determined previously to be infected with *P. olseni* and *P. marinus*, and plasmid-purified *P. chesapeaki* ITS DNA was first amplified with the *Perkinsus* genus-specific primers, Perk-ITS85/750, as described above and then digested with *Mbo* I restriction endonuclease. We chose to use only *P. marinus*, *P. olseni* and *P. chesapeaki* DNAs as controls, because these were the *Perkinsus* species, that we expected might be present in our samples. *Perkinsus olseni* was being used in the experiment and *P. marinus* and *P. chesapeaki* are common to Chesapeake Bay and potentially their DNAs could have contaminated the 1-µm filtered York River water, although this was not likely. PCR products were digested at 37 °C for 6 h and 10 µL of the digested PCR product was electrophoresed on a 3% agarose gel that was 1.5% agarose and 1.5% low melt agarose (Fisher Scientific, Raleigh, NC), stained with ethidium bromide and visualized under UV light. Images were recorded with an Alpha Imnotech FlourChem (San Leandro, CA) imaging system.

#### Histological Analysis

Tissues preserved for histological analysis in Davidson's solution (Shaw & Battle 1957) were dehydrated in a series of ethanol baths, infiltrated with paraffin and embedded in paraffin blocks prior to sectioning. Sections of 5-µm thickness were stained with Harris-hematoxylin and eosin. Histological sections of infected oysters were examined using light microscopy to visualize *Perkinsus* sp. parasite cells *in situ*.

#### In-situ Hybridization

Representative tissue sections from a few of the *C. ariakensis* oysters determined to have *P. marinus* or mixed infections of both *P. marinus* and *P. olseni* by species-specific PCR were evaluated by in situ hybridization (ISH). A genus-specific 5' end digoxigenin-labeled *Perkinsus* probe (Elston et al. 2004) was used to specifically target SSU rRNA sequences. *Perkinsus* species-specific probes, PmarLSU-181DIG (5'-GACAAACGGCGAACGACTC-3'), specific to *P. marinus*, and PolsLSU-464DIG (5'-CTCACAAAGT-GCCAAACAAGT-3'), specific to *P. olseni*, were designed by locating unique regions in aligned available *Perkinsus* species LSU rRNA gene sequences. Digoxigenin-labeled oligos were obtained from Operon Biotechnologies, Inc. (Huntsville, AL). The protocol followed for ISH was that previously published (Stokes & Bureson, 1995) with the modifications published by Elston et al. (2004). Pronase at a final concentration of 125 µg mL<sup>-1</sup> was used for permeabilization during a 30-min incubation, and a probe concentration of 7 ng µL<sup>-1</sup> was used for hybridization. The species-specific probes were tested for specificity with numerous *Perkinsus* sp.-infected reference tissues, including *P. marinus* in *C. virginica*, *P. chesapeaki* in *Mya arenaria* Linnaeus, 1758 and *P. olseni* in *Haliotis laevis* Donovan, 1808. Controls for each *Perkinsus* species-specific probe were tested identically except that they received hybridization buffer lacking probe during the hybridization step.

TABLE 1.

RFTM ranking and PCR-based *Perkinsus* genus-specific assay screening results of the challenge study oysters that were notched and inoculated with either SASW or cultured *P. olseni* cells and sampled from the experimental aquaria on days 44, 59 and 72.

RFTM Ranking	Day 44 (n = 2) SASW		Day 44 (n = 4) <i>P. olseni</i>		Day 59 (n = 2) SASW		Day 59 (n = 4) <i>P. olseni</i>		Day 72 (n = 6) SASW		Day 72 (n = 15) <i>P. olseni</i>	
	# RFTM	# PCR Pos	# RFTM	# PCR Pos	# RFTM	# PCR Pos	# RFTM	# PCR Pos	# RFTM	# PCR Pos	# RFTM	# PCR Pos
None (N)	2	1	2	1			1	1	3	3	1	1
Rare (R)									3	3	1	1
Very Light (VL)			1	1							2*	2
Light (L)			1	1			2	2			6	6
Light/Moderate (LM)											1	1
Moderate (M)					1	1	1	1				
Moderate/Heavy (MH)					1	1						
Heavy (H)											1	1
Very Heavy (VH)											3	3

\* Species-specific PCR assays indicated that one of these individuals, as well as one *P. olseni*-inoculated individual collected on day 21 (data not shown), contained DNA from both *P. marinus* and *P. olseni*. Only *P. marinus* DNA was detected in all other individuals.

## RESULTS

### Genus-specific PCR and RFTM Assay Results

All tissue samples used for DNA extraction yielded high quality genomic DNA as indicated by strong 1,800 bp amplification products that were clearly visible by UV illumination of agarose gels following PCR with the SSU rRNA gene universal primers. *Perkinsus* genus-specific PCR-based diagnostic screening of an initial baseline subset of 20 *C. ariakensis* sacrificed on January 24, 2005 showed that all animals were free of *Perkinsus* DNA. After being held in the laboratory aquaria for 59 days, prior to inoculation, five *C. ariakensis* were sacrificed and genus-specific PCR screening indicated a 100% prevalence of *Perkinsus* sp. DNA.

RFTM and *Perkinsus* genus-specific PCR screening results for the *C. ariakensis* that were inoculated with SASW or *P. olseni* cells are shown in Table 1. RFTM data were not collected during the first day of subsampling (Day 21), however the PCR screening indicated the presence of *Perkinsus* sp. DNA in both oysters sampled from those inoculated with SASW and in three of the four oysters sampled from the *P. olseni* injections. There were no *Perkinsus* cells observed by RFTM assays in the day 44 SASW sample, although *Perkinsus* sp. DNA was found by the PCR assay in one of the two oysters sampled. Very light-light RFTM rankings were found for two of the four *P. olseni*-inoculated oysters sampled on day 44 and three of the four were positive in the *Perkinsus* genus-specific PCR assay. On day 59 both of the SASW-inoculated oysters sampled had moderate-moderate/heavy *Perkinsus* sp. tissue burdens and *Perkinsus* sp. DNA was found in both by the PCR assay. Three of the four *P. olseni*-inoculated oysters that were sampled on day 59 had light-moderate infections as indicated by the RFTM assay and *Perkinsus* DNA was detected in all four of the oysters. On day 72, when the experiment was terminated, six SASW- and 15 *P. olseni*-treated oysters remained and were sacrificed. *Perkinsus* DNA was found by the PCR assay in all of the oysters remaining from the two treatment groups (Table 1). *Perkinsus* sp. cells were not detected, however, by the RFTM assay in three of the SASW-inoculated or in one of the *P. olseni*-inoculated oysters. The other three remaining SASW-inoculated oysters had rare RFTM rankings. Rare-light/moderate

tissue burdens were found in nine *P. olseni*-inoculated oysters at the end of the experiment and four *P. olseni*-inoculated oysters had heavy-very heavy infections as indicated by the RFTM assay.

RFTM and *P. marinus* PCR assay results for the 29 untreated *C. ariakensis* that were still alive and were sacrificed on day 37 of the challenge experiment are shown in Table 2. Eighteen of these oysters were ranked as having rare to light *Perkinsus* sp. tissue burdens, two were ranked as light/moderate and two as moderate-moderate/heavy tissue burdens. Seven had no observable *Perkinsus* sp. cells in the RFTM assay, however five of these were positive in the PCR assay. *Perkinsus marinus* DNA, as indicated by the PCR assay, was found in 23 of these 29 untreated oysters (Table 2), with no DNA detected in three of the oysters with a rare RFTM ranking and in one with a light infection as indicated by the RFTM assay.

### Mortality Data

Mortality was observed in untreated oysters and in those injected with either SASW or *P. olseni* (Table 3). For the untreated *C. ariakensis*, two died during the 37 day period that they were

TABLE 2.

RFTM ranking and PCR-based *P. marinus* assay screening results of control untreated *C. ariakensis* (unnotched and uninoculated) on day 37 of the challenge study.

RFTM Ranking	Day 37 (n = 29)	
	# Individuals	# <i>P. marinus</i> PCR+
None (N)	7	5
Rare (R)	7	4
Very Light (VL)	3	3
Light (L)	8	7
Light/Moderate (LM)	2	2
Moderate (M)	1	1
Moderate/Heavy (MH)	1	1
Heavy (H)		
Very Heavy (VH)		

(Note: *P. olseni* DNA was not detected in any of these oysters.)

TABLE 3.

Daily observed *C. ariakensis* mortalities during the course of the challenge experiment.

Day	Untreated	25 ppt SASW	<i>Perkinsus olseni</i>
2	1		
11	1		
37		1	
43	na		1
49	na	4	1
53	na		1
55	na		4
58	na	1	1
59	na	1	
64	na	1	2
66	na		1
72	na		6

na = not applicable because the oysters from the untreated tank were sacrificed on day 37.

Column headings indicate the sample treatments.

held in the aquaria. For the SASW treatment and the *P. olseni* treatment, cumulative mortality after 72 days was 40.0% and 46.2%, respectively. Of the 27 dead *C. ariakensis* removed from the untreated tank and the experimental aquaria during the course of the experiment, it was possible to conduct RFTM analysis on 11 oysters and PCR analysis on 22. All tissues taken from dead *C. ariakensis* were PCR positive for *Perkinsus* sp. DNA. Of these 11 oysters examined by RFTM, 2 had none, or rarely observable *Perkinsus* sp. cells, 2 had very light or light tissue burdens, 1 had a light/moderate tissue burden and 6 had moderate to heavy tissue burdens of *Perkinsus* sp. cells.

#### Species-specific PCR Screening for *Perkinsus marinus* and *Perkinsus olseni*

DNAs from all oysters shown to be PCR positive with the *Perkinsus* genus-specific assay were analyzed using both *P. marinus*-specific and *P. olseni*-specific primers. Because the *P. olseni* primers were new for this study, specificity was tested against *P. marinus* and *P. chesapeaki* DNAs. The *P. olseni* primers did not amplify DNA from these other *Perkinsus* species. Sequencing of amplification products further confirmed the specificity as sequences of amplification products from all positive *P. olseni*-specific reactions matched those of GenBank deposited *P. olseni* (*P. atlanticus*) sequences. The five *C. ariakensis* taken as a baseline sample immediately prior to the start of the study, and all untreated oysters had only *P. marinus* DNA. In addition, all but two of the *C. ariakensis* that were SASW- or *P. olseni*-inoculated and that were *Perkinsus* sp. positive with the genus-specific primers, were positive for only *P. marinus* DNA. Two oysters that were inoculated with *P. olseni* at the start of the challenge and were sampled on days 21 and 72 were positive for both *P. marinus* and for *P. olseni* DNA. Tissue from all dead oysters collected from either the experimental or untreated aquaria were found to be PCR-positive for *P. marinus* only.

#### RFLP Results

The *P. olseni* and *P. marinus* positive control DNA isolated from cultured cells, and plasmid-purified *Perkinsus chesapeaki*

ITS region DNA, were amplified in the *Perkinsus* genus-specific assay and were digested with *Mbo* I restriction endonuclease. Results confirmed that *P. marinus* ITS region DNA has a unique digestion profile when digested with *Mbo* I and the observed fragment sizes of approximately 17 bp, 226 bp and 264 bp were consistent with the fragment sizes expected for *P. marinus*.

#### Histology

Ten animals shown by PCR to have *Perkinsus* sp. DNA, and whose tissues showed light to very heavy RFTM rankings were chosen for further histological examination. A tissue section from one animal with a very heavy RFTM ranking that had died during the experiment showed gross lesions (Fig. 1A) and observable parasite cells when stained with Harris-hemotoxylin and eosin (Fig. 1B). Parasite cells were numerous and observed systemically

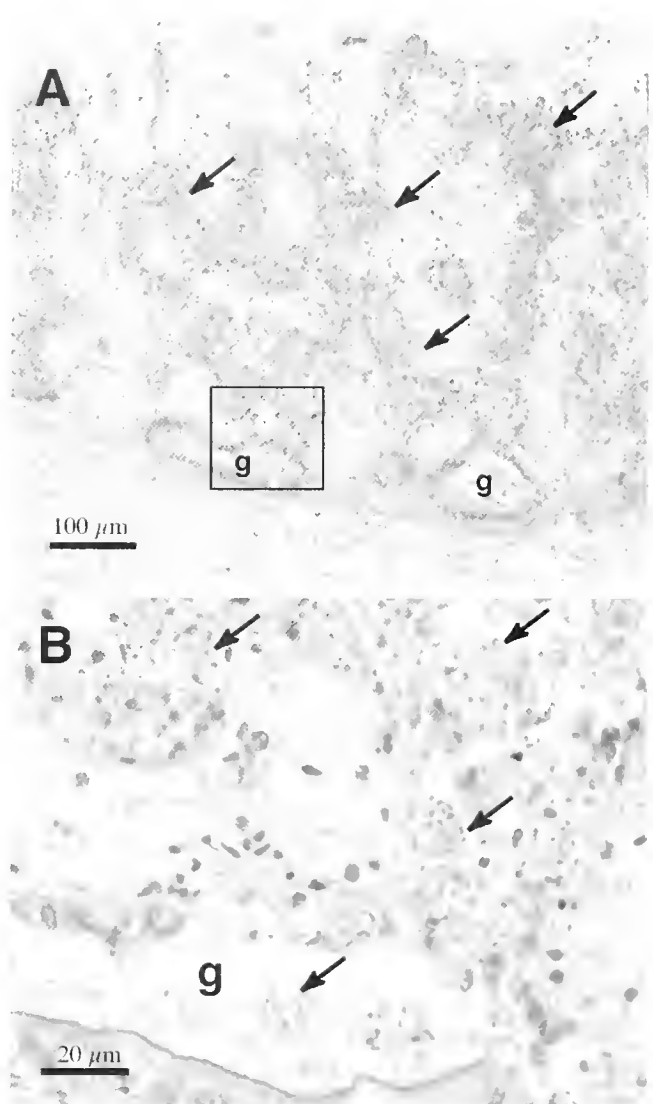


Figure 1. H&E-stained histological section of *Perkinsus marinus* lesions in the gonad of *Crassostrea ariakensis*. (A) Low power photomicrograph showing the extent of the lesions (arrows) in the vicinity of the gonoduct (g). Square represents the area shown in B. (B) Higher magnification in the vicinity of a gonoduct (g) showing *P. marinus* cells (arrows) in the lesions.

throughout the visceral mass. Obvious lesions caused by *Perkinsus* sp. infection were not observed by histology in tissue sections taken from animals with light or moderate *Perkinsus* sp. tissue burdens as determined by the RFTM assays.

#### ISH Assay Results

*In-situ* hybridization probes designed to target the LSU rRNA genes of *P. marinus* or *P. olseni* exhibited specificity for the targeted *Perkinsus* species. In the specificity tests, the *P. marinus* probe hybridized only to the *Perkinsus* cells in the sample of *P. marinus* in *C. virginica* and the *P. olseni* probe hybridized to *Perkinsus* sp. cells in the control sample of *P. olseni* in *Haliotis laevis*. The probes did not cross-react with nontargeted *Perkinsus* species or host tissues in other control samples. Figure 2A to D shows four consecutive sections from the heavily infected oyster shown in Figure 1. Figure 2A is an H&E-stained section. Figure 2B shows positive *in-situ* hybridization with the PmarDIGLSU-181 probe to *Perkinsus* sp. cells indicating that the lesions were caused by *P. marinus*. The *P. marinus*-specific probe bound to cells throughout the digestive epithelium, gonads and gonoducts. *In situ* hybridization reactions conducted without probe (Fig. 2C), or with the PolsDIGLSU-464 probe (Fig. 2D), produced no signal in host tissues of this oyster. The PolsDIGLSU-464 probe was also tested against two individuals that were inoculated with *P. olseni* and found to harbor both *P. marinus* and *P. olseni* DNA by the PCR assays. The *P. marinus* probe hybridized to *Perkinsus* sp. cells in the dually infected oysters, however, no binding of the *P. olseni* probe was observed, suggesting that infections by *P. olseni* were extremely light, or that only *P. olseni* DNA was present and not viable cells.

#### DISCUSSION

Initial screening using a PCR-based diagnostic method on a sample of the triploid *C. ariakensis* oysters that were obtained for the challenge study suggested that they did not harbor *P. marinus*. A small sample ( $n = 5$ ) of these oysters screened 59 days later, however, had 100% *P. marinus* DNA prevalence as indicated by the *P. marinus*-specific PCR assay. We suggest that the *C. ariakensis* oysters either acquired a small number of *P. marinus* cells that were not numerous enough to be detected by our initial PCR assay of gill/mantle tissue, while in the VIMS hatchery before collection for this experiment, or while being held in unfiltered York River water for 4 days prior to being brought into the aquaria where they were subsequently held only in filtered York River water. The *P. marinus* proliferated to PCR detectable levels after 59 days in aquaria with disease developing and mortality subsequently occurring in not only those oyster groups that had been subjected to notching and inoculation with either SASW or *P. olseni* for the challenge study, but also in the control group of unnotched and untreated oysters that were simply held for an additional 37 days during the challenge experiment.

RFTM and PCR assay results for the *C. ariakensis* initially inoculated with either 25 ppt SASW or cultured *P. olseni* cells for the challenge study revealed a progression of *P. marinus* infection in the oyster tissues. Sampling and subsequent PCR-based screening of both the 25 SASW and *P. olseni*-inoculated oysters on Days 21 and 44 indicated that *P. marinus* DNA was found in 83.3% and 66.7% of the oysters, respectively. We believe that the apparent decrease in *P. marinus* prevalence on day 44 reflects sampling error related to the small sample sizes ( $n = 6$ ) assayed to determine PCR-based *Perkinsus* sp. prevalence at each time

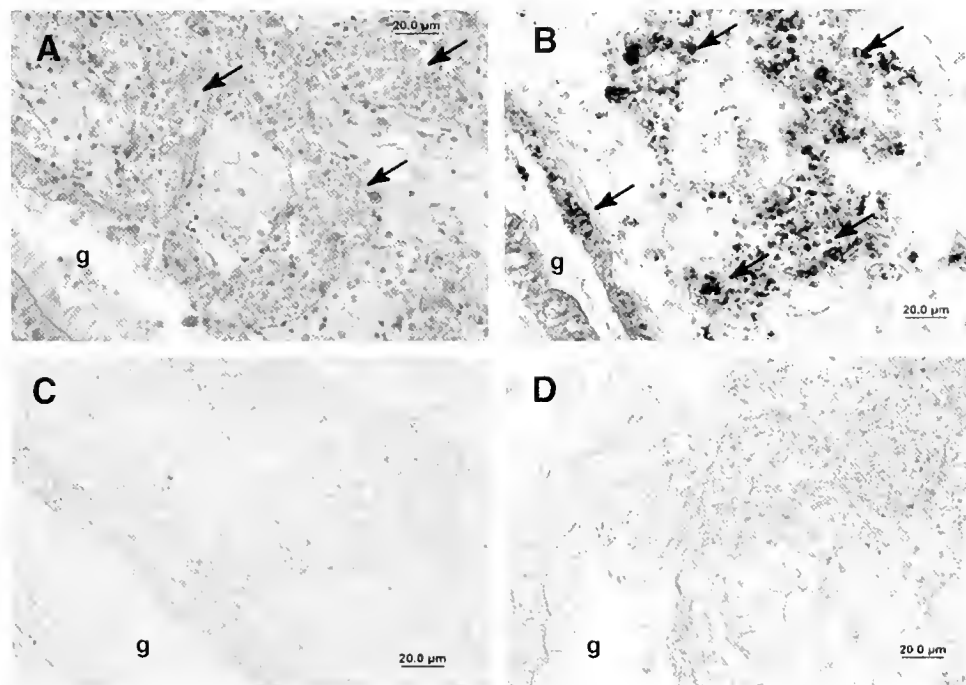


Figure 2. *Perkinsus marinus* in *Crassostrea ariakensis*. (A) H&E-stained histological section in the vicinity of a gonoduct (g) showing *Perkinsus marinus* lesions (arrows). (B) *In situ* hybridization with the *P. marinus*-specific probe, Pmar181LSUDIG, illustrating strong binding to the *Perkinsus* sp. cells (arrows). (C) No probe negative control showing no binding to the *C. ariakensis* tissue. (D) *In situ* hybridization with the *P. olseni*-specific probe, Pols464LSUDIG, illustrating no binding to the *Perkinsus* sp. cells.

point. Although *P. olseni* DNA was found in two of the *P. olseni*-inoculated oysters (one each on days 21 and 72), there was an increase in the observed PCR prevalence of *P. marinus* DNA to 100% in samples of all treated oysters collected on days 59 and 72. Likewise, as determined by RFTM assays, the tissue burdens for the two positive individuals were light in the *P. olseni*-treated oysters collected on day 44 of the experiment. However, among samples of these same oyster groups that were collected on days 59 and 72, not only light infections, but also moderate to very heavy tissue burdens were observed. Interestingly, disease progression in those *C. ariakensis* not notched or inoculated, mimicked that seen in the manipulated experimental oysters based on the RFTM-based tissue burdens. By day 37 when the control oysters were sacrificed, the observed *Perkinsus* sp. tissue burdens ranged from light to moderate/heavy.

During the course of the experiment, there were many instances where dead oysters were discovered in the various treatment groups, with little or no tissue available for either PCR or RFTM analysis. It is important to note, however, that of the 1 dead oysters with available tissue, more than half (55%) had moderate to heavy tissue burdens.

Prevalence and intensity of *P. marinus* infections in *C. virginica* oysters are closely linked to temperature and salinity, and the rate of infection in the natural environment is believed to be proportional to the number of waterborne infective cells (Andrews & Hewatt 1957, Chu et al. 1994). Warm water temperatures, 20°C to 25°C, and high salinity >15 ppt, have been shown to correlate with times of maximal proliferation of parasite cells, as well as highest incidences of infection in oysters (Andrews & Hewatt 1957, Chu et al. 1994). Mortality of infected oyster hosts depends on the level of infection, with an apparent dose of between 10 and 100 cells adequate to establish infection (Chu 1996). Because of the effects of salinity and temperature on the parasite, the infections caused by *P. marinus* are seasonal in the Chesapeake Bay, with maximum parasite prevalence recorded in late summer and minimum prevalence observed during the winter months (Burrenson & Ragone Calvo 1996). York River water conditions during the time that the oysters were in the holding tank were approximately 9°C and 16 ppt salinity. Environmental conditions of the York River were not likely favorable for *P. marinus* proliferation at the start of these experiments; however, it has been shown that parasite cells can remain viable in over wintering conditions of temperatures as low as 4°C and a salinity of 4 ppt (Chu & Greene 1989, Ragone Calvo & Burrenson 1994, Chu 1996). Therefore, we cannot discount the possible presence of parasite cells in the York River water during the holding period, when the oysters were in unfiltered water. The experimental conditions under which the oysters were held, 20°C and 25 ppt salinity, would have favored the proliferation of *P. marinus* cells present in the oysters, accounting for the increase in infection prevalence observed after the baseline sampling.

Cumulative mortalities were highest in those treatments where oysters were subjected to the extra stress of notching and injection, and the highest *Perkinsus* sp. tissue burdens were seen in dead *C. ariakensis*. We may have observed higher cumulative mortalities

in the untreated control oysters, as well, had they been held for a longer time period. In *C. virginica*, digestive gland epithelia and the stomach are often heavily colonized with *P. marinus* and damaged. As parasite proliferation increases to lethal levels, massive tissue sloughing occurs, which eventually contributes to the death of the animals (Mackin, 1951). In this study, tissue sections from a moribund *C. ariakensis* with a very heavy RFTM ranking showed dense, systemic *P. marinus* infection and the ISH assays confirmed that all of the observable *Perkinsus* sp. cells were *P. marinus*. Collectively, the results presented here strongly suggest that *P. marinus* was an important contributing factor to the death of these oysters.

Because *P. olseni* was found during a recent survey of *C. ariakensis* populations in Asia (Moss & Reece 2005), this study was initiated to examine the pathogenicity of *P. olseni* to *C. ariakensis*. At the termination of this experiment, however, PCR based diagnostics suggested that a relatively low number of the *P. olseni*-inoculated *C. ariakensis* ( $n = 2$ ) harbored both *P. marinus* and *P. olseni* DNA. This result may indicate that *C. ariakensis* are not readily susceptible to *P. olseni*, or that virulence attenuation of the parasite may have occurred during the culturing period prior to the use of the parasite as inoculum. Virulence attenuation with *in-vitro* cultured *P. marinus* cells has been documented (Ford et al. 2002). Recent studies, however, have demonstrated that cultured cell virulence is enhanced by supplementing media with host oyster homogenate (MacIntyre et al. 2003, Earnhart et al. 2004) and therefore, we plan to do homogenate supplementation of media for *Perkinsus* sp. isolates used in future challenge studies.

Although little data on *P. olseni* pathogenicity was obtained during this experiment, it provided valuable information regarding the potential for advanced *P. marinus* infections to occur in *C. ariakensis*. Prior field studies conducted in Chesapeake Bay have indicated that although *C. ariakensis* is capable of acquiring *P. marinus* infections (Calvo et al. 2001), there was no evidence that *C. ariakensis* was susceptible to the advanced parasite infections known to occur in *C. virginica*. We contend that the experimental environmental conditions, under which the oysters were held for a total of 5 months, combined with the stress of the unnatural aquaria environment and experimental manipulation, may have promoted the development of the intense *P. marinus* infections in *C. ariakensis* that were observed here. This study provides valuable information on potentially problematic disease issues including parasite proliferation that can arise if *P. marinus*-infected *C. ariakensis* encounter stress challenges in the wild or aquaculture environment, or if they are held in hatcheries or laboratories under stressful conditions.

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## AGE AND GROWTH OF WILD SUMINOE (*CRASSOSTREA ARIAKENSIS*, FUGITA 1913) AND PACIFIC (*C. GIGAS*, THUNBERG 1793) OYSTERS FROM LAIZHOU BAY, CHINA

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**ABSTRACT** Shell height at age estimates from Summoe (*Crassostrea ariakensis*) and Pacific (*C. gigas*) oysters from a natural oyster reef in Laizhou Bay, China were compared with shell height at age estimates from triploid *C. ariakensis* of known age from the Rappahannock River, Virginia. *C. ariakensis* and *C. gigas* reach shell heights in excess of 76 mm (3 inches) within 2 years after settlement regardless of the source location. This fast growth appears to continue through at least age 4 or age 5 in wild individuals, because the growth trajectories for both species had not reached asymptotic height in the oldest individuals collected. Estimates of the asymptotic maximum height ( $SH_{max}$ ) from fitted Von Bertalanffy (VB) growth models were greatest for Chinese *C. ariakensis* (244.0 mm, standard error of the mean [SE] 30.4) and near the maximum shell height (227.0 mm) measured at the time of collection. Maximum shell heights measured on live Chinese *C. gigas* (173.0 mm) and Rappahannock *C. ariakensis* (190.0 mm) were also within the standard error estimates for the  $SH_{max}$  estimates from the fitted VB models for Chinese *C. gigas* (158.6 mm, SE 20.3) and Rappahannock *C. ariakensis* (183 mm, SE 19.1). Fitted VB growth curves were not significantly different between species within the same habitat, within species in different habitats or between species in different habitats. The ratio of shell height to shell width and shell height to shell inflation for triploid *C. ariakensis* was significantly less than similar ratios observed in wild *C. ariakensis* and *C. gigas* oysters.

**KEY WORDS:** Summoe oyster, *Crassostrea ariakensis*, Pacific oyster, *Crassostrea gigas*, age determination, bivalves, oysters, growth rates, resilium

### INTRODUCTION

Bivalves carry a complete record of their lives in their shells (Pannella & MacClintock 1968, Lutz & Rhoads 1980, Richardson 2001). Environmental changes as well as seasonal changes in biology and physiology are recorded in the shell structure as external rings, internal lines and growth increments or both (e.g., Pannella & MacClintock 1968, Lutz & Rhoads 1980, Ropes 1985, Richardson 2001). Using the terminology of Richardson (2001), a growth line is an internal line deposited once a year (annually), whereas the annual growth increment is the distance separating adjacent growth lines. In oysters, internal growth lines may be observed in the resilium of the hinge structure (Richardson et al. 1993a, 1993b, Kirby et al. 1998, Richardson 2001) and in the middle homogenous shell layers. The resilium is protected from damage or erosion by the valves. During periods of reduced shell growth, the ligament growth also slows producing growth lines in both shell and resilium (Richardson 2001). In temperate oysters, the annual growth cycle includes one thick, grey growth line deposited in between white growth increments (Richardson et al. 1993a). Enumerating the thick grey growth lines within a resilium or shell cross section provides an estimate of age (years) for the oyster (Richardson et al. 1993a, 1993b, Kirby et al. 1998, Richardson 2001).

Pacific (*Crassostrea gigas*, Thunberg 1793) oysters are cultured for commercial purposes around the world with initial introductions often made to supplement native oyster stocks (see reviews by Mann 1981, Mann et al. 1991, Shatkin et al. 1997). In recent years, Summoe (*C. ariakensis*, Fugita 1913) oysters have also been proposed as candidates for commercial aquaculture and/or introductions to supplement native oyster stocks (e.g., Langdon & Robinson 1996, Hallerman et al. 2002). Whereas little is known about the population age structure of wild populations of either species in natural reef settings in their native Asian waters, some

data on growth rates in native waters are available. Zhang and Lou (1956, reported by Zhou & Allen 2003) report Chinese *C. ariakensis* reaching sizes of 100–160 mm shell height in 2–3 y, whereas Fujimori (1929, reported by Cahn 1950) describes Japanese *C. ariakensis* reaching 197-mm shell height at ages of 6 y; *C. ariakensis* from China and Japan are described as large reaching sizes in excess of 200 mm shell height (Torigoe 1981).

The observed morphology of these oysters is variable. The morphology of *C. ariakensis* has been described as discoid (Ahmed 1971) and oval or rounded with relatively flat shell layers with only the left valve concave (Wakiya 1929, Torigoe 1981). Wakiya (1929) describes shells of *C. ariakensis* from soft mud habitats as “extremely elongated” and difficult to distinguish from *C. gigas* found on mud bottoms. *C. gigas* are also described as large (reaching shell heights >400 mm, Torigoe 1981) but both valves are concave with rippled shell layers (Torigoe 1981, Langdon & Robinson 1996). Torigoe (1981) describes *C. gigas* as “oval to spatulate,” but Wakiya (1929) describes adult *C. gigas* as “extremely elongated”.

The objectives of this study are to describe shell morphology, shell height at age relationships and growth curves for wild *C. ariakensis* and *C. gigas* specimens collected concurrently from a natural population of reef oysters in Laizhou Bay, Gulf of Bohai, China and compare these data with shell morphology, shell height at age relationships, and growth curves for triploid *C. ariakensis* grown in Chesapeake Bay, USA.

### METHODS

#### Sample Collection

Oysters of both species were collected from the same natural intertidal oyster reef in Laizhou Bay, Gulf of Bohai, China (37°14'38.0, 119°03'29.9) during a quadrat survey conducted in June 2004 by Dr. Mark Luckenbach (Virginia Institute of Marine Science (VIMS), Eastern Shore Laboratory) and Dr. Christopher Richardson (University of Wales Bangor, Menai Bridge). Ambient

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salinities were 30 ppt at the time of collection. Oyster shells were separated from meats in the field at the time of collection. Shells were labeled, bagged and shipped as valve pairs corresponding to individuals. The species co-occurred on the reef and were distinguished from each other by genetic analyses (mitochondrial 16S-based molecular key, per Banks et al. 1993) of tissue samples conducted by Dr. Ryan Carnegie (VIMS, Department of Environmental and Aquatic Animal Health). A total of 11 *C. ariakensis* individuals and 186 *C. gigas* individuals were identified from this collection on the basis of genetic analyses.

Four triploid *C. ariakensis* were collected from the lower Rappahannock River, Virginia during May 2004 as the remnant of a controlled field experiment conducted with aquacultured triploid *C. ariakensis* in 2001 (Dr. J. Wesson, Virginia Marine Resources Commission). These animals were spawned at VIMS in June 2000 (Dr. S. K. Allen, VIMS Aquaculture Genetics and Breeding Technology Center, pers. comm.); thus when they were collected and again certified as triploid in May 2004 they were approximately 4 years old.

#### Laboratory Methods

The hinge structure and shell morphology of each individual oyster was examined to evaluate suitability for sectioning and age estimation. Oysters in which the resilium, adductor muscle scar and growth edge on the left valve that formed a straight line were deemed suitable for aging and were set aside. A total of nine Chinese *C. ariakensis* and 19 Chinese *C. gigas* were suitable for estimation of age and growth rates. All four of the Rappahannock triploid *C. ariakensis* were used for age estimation.

The shells for estimation of age and growth rates were gently cleaned to remove attached epifauna using a sonicator. Measurements of shell height (SH, maximum dimension from the hinge to the growth edge, mm), maximum shell width (SW, maximum dimension perpendicular to the hinge across one valve, mm) and maximum shell inflation (SI, maximum dimension perpendicular to the hinge across both valves, mm) were made for each individual (Fig. 1). The ratios of (1) SH to SW and (2) SH to SI were calculated for each individual as metrics to describe shell shape. SH:SW ratios near 1 indicate a disk shaped or round individual, whereas SH:SW ratio values  $>2$  are indicative of individuals that are long and narrow. SH:SI ratios provide an index of cupping or depth. SH:SI ratio values near 1 indicate an individual that is as deep or cupped as it is long (a spherical oyster), whereas higher SH:SI values describe oysters that are longer than they are deep. SH:SW and SH:SI ratios were compared across species within a site (Chinese *C. ariakensis* vs. Chinese *C. gigas*) and across sites within a species (Chinese vs. Rappahannock *C. ariakensis*) using 1-way ANOVAs with significance values set at  $P = 0.05$  a priori.

Shells were sectioned from hinge to the growth edge along a straight line that included the resilium and adductor muscle scar (the major axis of growth, Fig. 1 (a)) using a diamond blade saw. Shell cross-sections were polished using wettable carborundum disks (240 through 600 grit) and diamond polishing suspension (Buehler Metadai, 6 and 1  $\mu$ m suspension). Shells were allowed to dry thoroughly after polishing before examination for growth signatures.

The section of the left valve containing the bulk of the adductor muscle scar was used for shell cross-section evaluation. The number of growth lines within each shell cross section were counted by following the thick grey lines that were continuous from the hinge

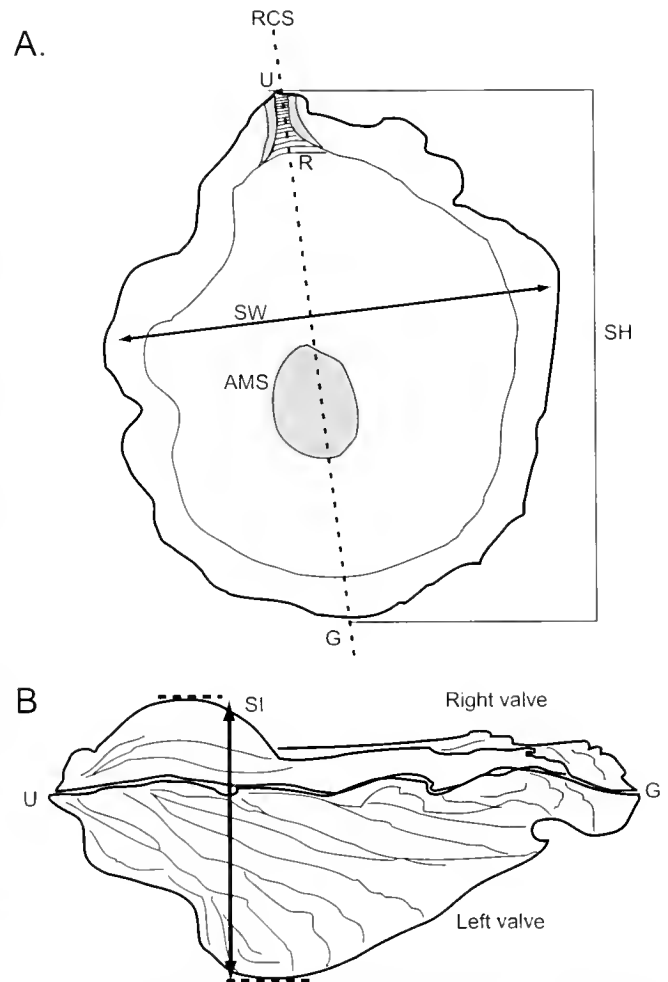


Figure 1. (A) Sketch of the left valve of a *Crassostrea* shell with the umbo (U), resilium (R), and adductor muscle scar (AMS) shown as reference points. Morphological measurements made for each shell are shown including shell height (SH, mm), shell width (SW), and shell inflation (SI). After morphological measurements were made, a radial cross section (RCS) was made for each shell following the growth trajectory through the resilium (R) and AMS to the growth edge (G). (B) Sketch of a valves of a *Crassostrea* shell in profile showing where the measurement for shell inflation (SI) was made.

to the outer shell layer throughout the valve radial cross section (Fig. 2a). Lines visible in the cross section had to also be visible in the resilium structure to be included (Fig. 2b). The position of growth lines along the radial cross section in relation to the resilium of the hinge ligament provides a growth curve for individual animals. The distance from the hinge to the emergence of a growth line in the shell exterior of the radial cross section was measured (mm) for each shell. The resilium was used to validate cross section lines given the foliation and ornamentation found in the shells of larger specimens. Shell height (mm) at age (yr) curves were plotted for populations based on the sequential measurements of growth lines (height at age) made from left valve cross sections from a size range of individuals.

Population growth curves (age (yr), shell height (mm)) were fitted using the von Bertalanffy (VB) model (von Bertalanffy 1938) with nonlinear least squares regression. This model describes maximum growth and does not assume rotational symmetry about an inflection point (Brown & Rothery 1993). It has been

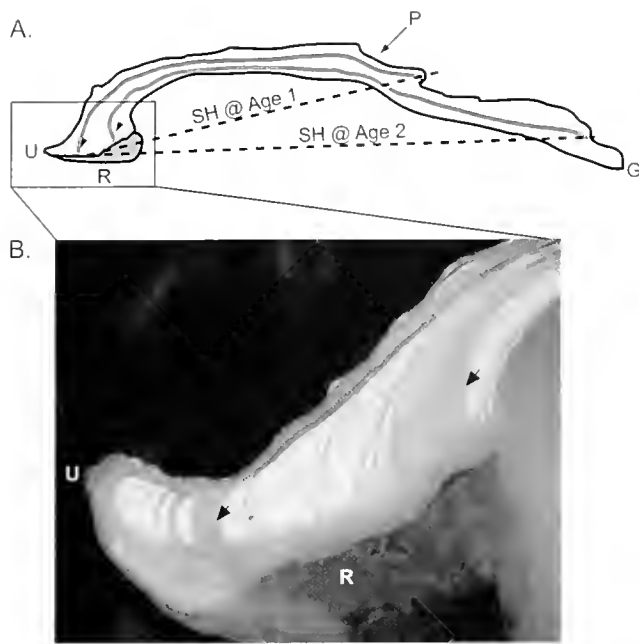


Figure 2. A. Sketch of a radial cross section of a *Crassostrea* left valve showing the annual growth lines (black arrows). Shell height at age measurements were made along axis U-G from the umbo to locations where the annual growth lines ended at the periostracum (P). B. Photograph of a representative resilium radial cross section from a 2-year-old *C. gigas* with the annual growth lines marked (black arrows).

used extensively to describe the growth of other species of shellfish (e.g., *Spisula solidissima*, Sephton & Bryan 1990; *Rangia cuneata*, Fritz et al. 1990, *Mercenaria mercenaria*, Jones et al. 1990, Devillers et al. 1998, *Ostrea edulis*, Richardson et al. 1993a and *Tiostrea* (= *Ostrea*) *lunata*, Richardson et al. 1993b). The model equation is:

$$SH_t = SH_{max} (1 - e^{-k(t-t_0)})$$

where  $SH_t$  is the shell height at time  $t$ ,  $SH_{max}$  is the maximum or asymptotic shell height,  $t_0$  is the size at time 0, and  $k$  is a rate constant.

The fitted VB growth curves for populations of Chinese *C. ariakensis*, Chinese *C. gigas*, and cultured Rappahannock *C. ariakensis* were compared as pairs using the nonlinear coincident curve method described by Haddon (2001) based on Chen et al. (1992) and Zar (1996). This method compares two curves using the analysis of the residual sum of squares to test if two or more nonlinear curves are statistically different (Haddon 2001).

## RESULTS AND DISCUSSION

### Morphology

*C. ariakensis* from the Rappahannock River were more cupped than similarly sized individuals of either species from China as indicated by inflation measurements and SH:SI ratios (Table 1, Fig. 3, Fig. 4). The triploid Rappahannock River individuals were discoid (plate-like) when viewed from above as indicated by SH:SW ratios near 1 (Table 1, Fig. 3, 4). Ratios of SH:SW and SH:SI for Rappahannock *C. ariakensis* were significantly less than the same ratios for Chinese *C. ariakensis* (ANOVAs,  $DF = 1$ ,  $F = 28.1$  (SH:SW) and  $F = 8.86$  (SH:SI),  $P < 0.05$ ). Ratios of SH:SW and SH:SI from Chinese *C. ariakensis* and *C. gigas* were similar (ANOVAs,  $DF = 1$ ,  $F = 0.73$  (SH:SW) and  $F = 0.47$  (SH:SI),  $P > 0.40$ ). Ratios from all four species and site combinations satisfied assumptions of homogeneity and normality without transformation. The wild Chinese *C. ariakensis* and *C. gigas* were typically long (SH), narrow (SW), and flatter in profile (SI) with higher SH:SW and SH:SI ratios than the Rappahannock River *C. ariakensis* (Table 1, Fig. 3, 4). The descriptions and photographs of *C. ariakensis* provided by Cahn (1950) and Torigoe (1981) from Ariake Bay, Japan are similar in terms of shape (tongue-like) and SH:SW ratio to the large Chinese *C. ariakensis* and *C. gigas* examined in this study (Table 1, Fig. 3A to F, 4).

### Shell Height at Age

Chinese *C. ariakensis* and *C. gigas* displayed overlap in the range of observed shell heights at age with each other and with the triploid *C. ariakensis* from the Rappahannock River, Virginia (Table 2). *C. ariakensis* and *C. gigas* reach shell heights in excess of 76 mm (3 inches) within 2 years after settlement (Table 2, Fig. 5) regardless of the source location. This fast growth appears to continue through at least Age 4 (*C. gigas*, Fig. 5) and Age 5 (*C. ariakensis*, Fig. 5) in wild individuals because the growth trajectory for both species had not begun to flatten in the oldest individuals collected (Fig. 5). The observed shell height ranges for each age class (Table 2) are within the ranges of previously published reports of shell heights at age for both species (Table 3). The triploid *C. ariakensis* in the Rappahannock River 2001 to 2004 followed a growth trajectory similar to that reported for Ariake Bay Japan by Cahn (1950, from Fujimori 1929, Fig. 5). The Rappahannock animals had not reached asymptotic shell height when they were collected in May 2004 (Fig. 5).

The observed range in shell heights at Age 1 for the Chinese oysters may be the result of differences in the timing of individual

TABLE 1.

Summary of morphological measurements made on individual wild diploid *C. gigas* and *C. ariakensis* from Laizhou Bay, China and cultured triploid *C. ariakensis* from the Rappahannock River, USA. Measurements were made for shell height (SH), shell width (SW), and shell thickness or inflation (SI) as shown in Figure 1 and explained in the text. Ranges for each measurement type (SH, SW, SI) are presented for each species by habitat with average ratio and standard error of the mean (SE) values.

Site	Species	n	SH Range (mm)	SW Range (mm)	SI Range (mm)	Average SH:SW Ratio (SE)	Average SH:SI Ratio (SE)
Laizhou Bay, China	<i>C. ariakensis</i>	9	113–227	60–135	26.3–72.0	1.79 (0.07)	4.01 (0.30)
	<i>C. gigas</i>	19	38–173	35–107	25.0–50.0	1.92 (0.09)	3.74 (0.23)
Rappahannock River, US	<i>C. ariakensis</i>	4	170–190	145–157	55.1–79.6	1.19 (0.03)	2.57 (0.23)

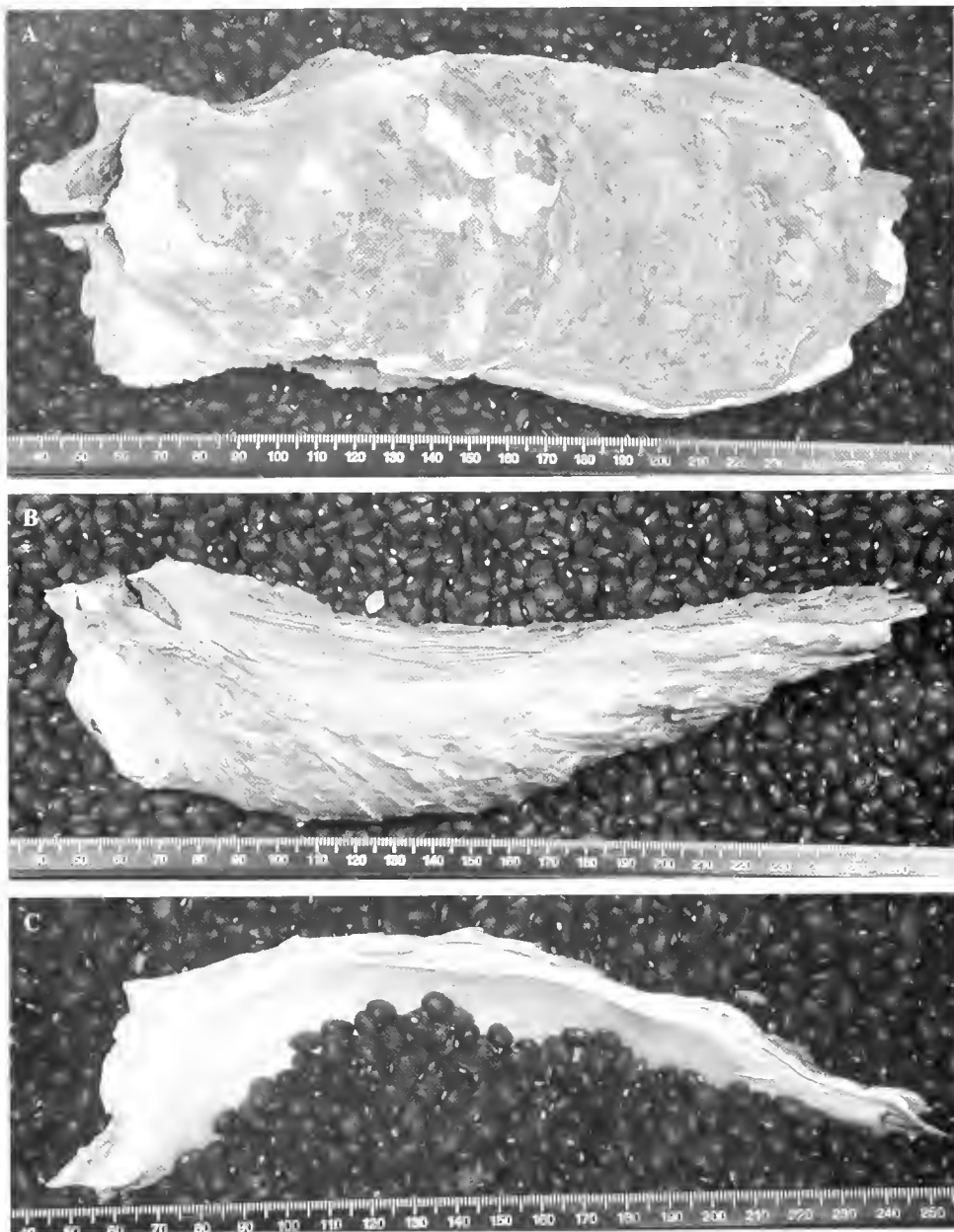


Figure 3. Photographs of representative specimens from China and the Rappahannock River, Virginia. A to C, Chinese *C. ariakensis*, SH = 227 mm.

recruitment. Potential differences in the timing of spawning (Perdue & Erickson 1984, Langdon & Robinson 1996) and recruitment between species may be offset by differences in the growth rates of each species at temperatures less than 25 °C. Whereas recruits of both species grow faster at 25 °C than at 15 °C, *C. gigas* spat growth is less at 20 °C than at 25 °C and *C. ariakensis* spat grow as fast at 20 °C as they do at 25 °C (Langdon & Robinson 1996). *C. gigas* that settle early in the season may stop growing earlier than conspecific *C. ariakensis* because of differences in thermal physiology. Although *C. ariakensis* may recruit later, they are able to grow at lower temperatures than *C. gigas* and the relatively extended growing window probably offsets differences in the timing of recruitment resulting in the observed overlap in size range (*C. gigas*: 27–55 mm SH, *C. ariakensis*: 27–57 mm SH) when the first growth line (Age 1) was deposited.

#### VB Growth Model Coefficients and Model Fitting

Fitted VB growth curves (Fig. 6) were not significantly different between species within the same habitat (Chinese *C. gigas* vs. *C. ariakensis*,  $F = 2.2$ ,  $P = 0.10$ , degrees of freedom = 71), within species in different habitats (Chinese *C. ariakensis* vs. Rappahannock *C. ariakensis*,  $F = 1.13$ ,  $P = 0.35$ ,  $df = 45$ ) or between species in different habitats (Chinese *C. gigas* vs. Rappahannock *C. ariakensis*,  $F = 0.93$ ,  $P = 0.43$ ,  $df = 55$ ).

Estimates of the asymptotic maximum height ( $SH_{max}$ ) were greatest for Chinese *C. ariakensis* (244.0 mm, SE 30.41) and near the maximum shell height (227.0 mm) measured at the time of collection (Fig. 6, Table 4). Maximum shell heights measured on live Chinese *C. gigas* (173.0 mm) and Rappahannock *C. ariakensis* (190.0 mm) were also within the standard error estimates for the

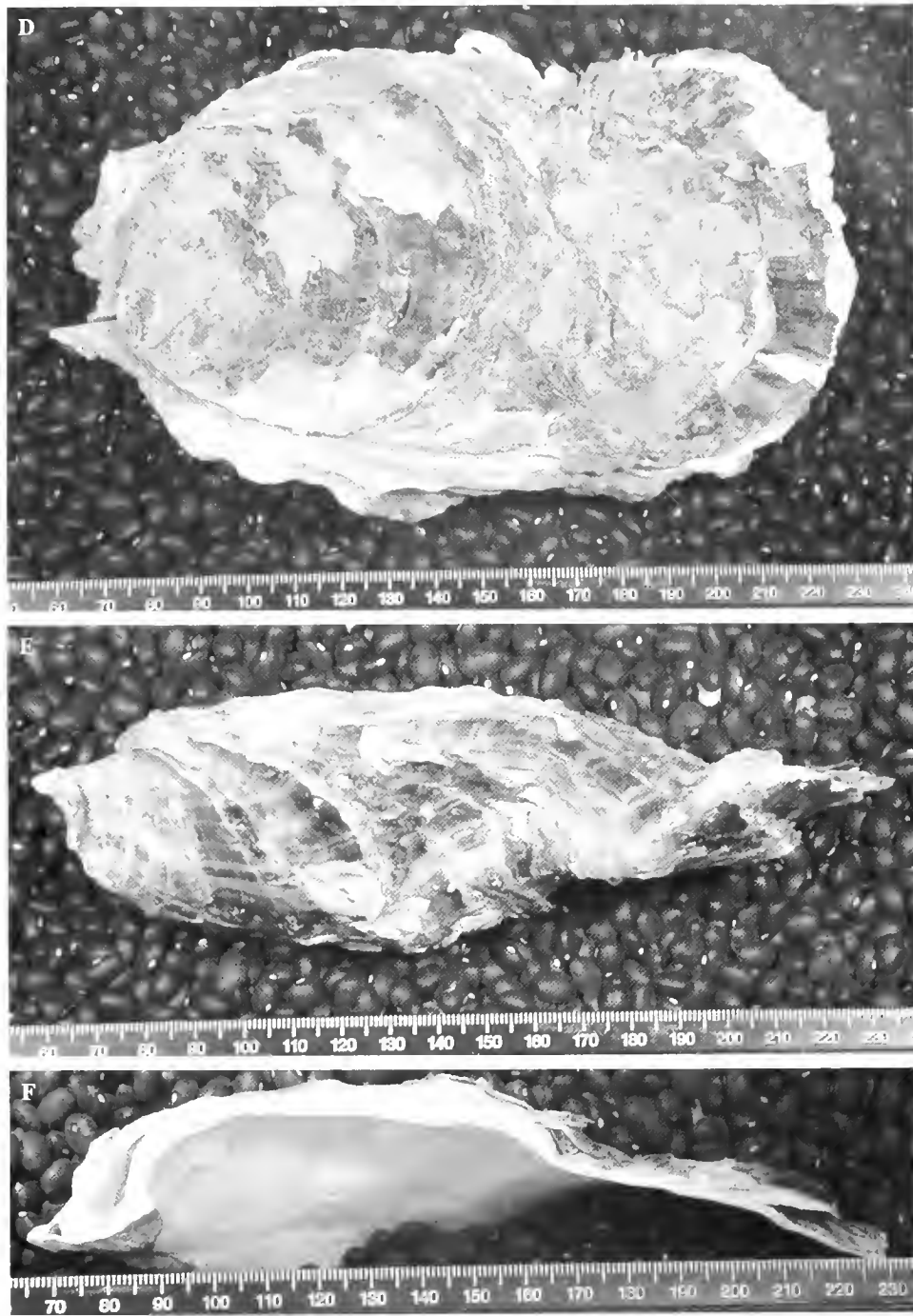


Figure 3. D to F, Chinese *C. gigas*, SH = 165 mm.

$SH_{max}$  estimates from the fitted VB models for Chinese *C. gigas* (158.6 mm, SE 20.25) and Rappahannock *C. ariakensis* (183 mm, SE 19.14; Table 4). The reported  $SH_{max}$  estimates for *C. ariakensis* (Table 4) are within the range of shell heights reported for *C. ariakensis* from Ariake Bay, Japan (Cahn 1950: 240 mm; Torigoe 1981: 200 mm). The  $SH_{max}$  estimates for *C. gigas* (Table 4) are smaller than the reported maximum size range for Japan (Cahn 1950: Tokoro, 400 mm; Torigoe 1981: Ariake Bay, 448 mm). The lack of representation of older age classes in the wild population and the fact that 13 out of the 19 *C. gigas* examined were 2 years old or less with only one 4 year old available resulted in dispro-

portionate representation of younger individuals and uneven sample sizes across the age distribution, which may have skewed the estimate of asymptotic height towards the smaller individuals (Fig. 5 and 6, Tables 2 and 4).

The  $k$  model parameter specifies the curvature of the fitted growth line (Gallucci & Quinn 1979) and is associated with the rate at which the organism approaches maximum size (Gallucci & Quinn 1979). Observed  $k$  values (Table 4) for *C. ariakensis* were 0.33 (Chinese, diploid) and 0.55 (Rappahannock triploid), respectively with a  $k$  value of 0.68 observed for the *C. gigas*. Coefficients of determination ( $R^2$ ) for the Chinese and Rappahannock *C. ari-*

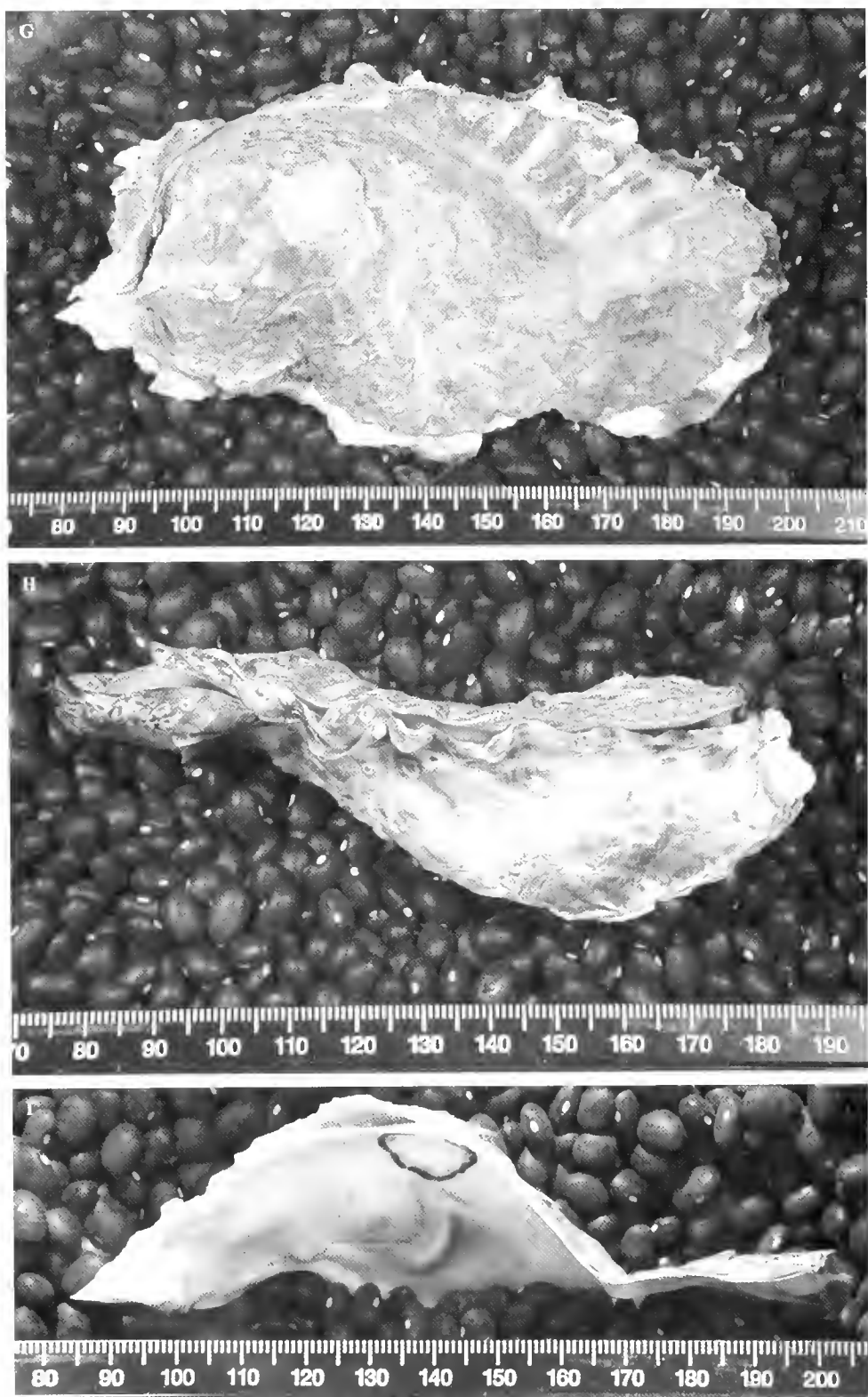


Figure 3. G to I, Chinese *C. gigas*, SH = 126 mm.

*akensis* were greater than or equal to 0.94 (Table 4). The *C. gigas* coefficient of determination was 0.89 and this may be reflective of the fact that most of the specimens examined (17/19) were less than 3 y old (Fig. 5, Tables 2 and 4). The relative under representation or absence of older *C. gigas* at or near asymptotic height is

reflected in the high  $k$  value (0.68) and lower coefficient of determination (0.89) relative to the *C. ariakensis* populations both of which had more balanced distribution of individuals across age classes (Fig. 5, Tables 2 and 4).

Salinities in Laizhou Bay (30 ppt) are higher than those typi-





Figure 3. J to L, Rappahannock *C. ariakensis*, SH = 180 mm.

cally observed in the lower Rappahannock River (12–15 ppt, Stroup & Lynn 1963), but both sites have salinities within the documented salinity tolerance of both species (e.g., Robinson 1992, Langdon & Robinson 1996, Almeida et al. 1997, Calvo et al. 1999, 2001, Grabowski et al. 2004). Seasonally water temperatures

in the Rappahannock River range from 4 °C to 28 °C (Stroup & Lynn 1963). Laizhou Bay probably experiences a similar annual water temperature range based on documented annual water temperature profiles from adjacent habitats (Yellow Sea, Chung et al. 1993; South Korean coastal habitats, Kang et al. 2000).

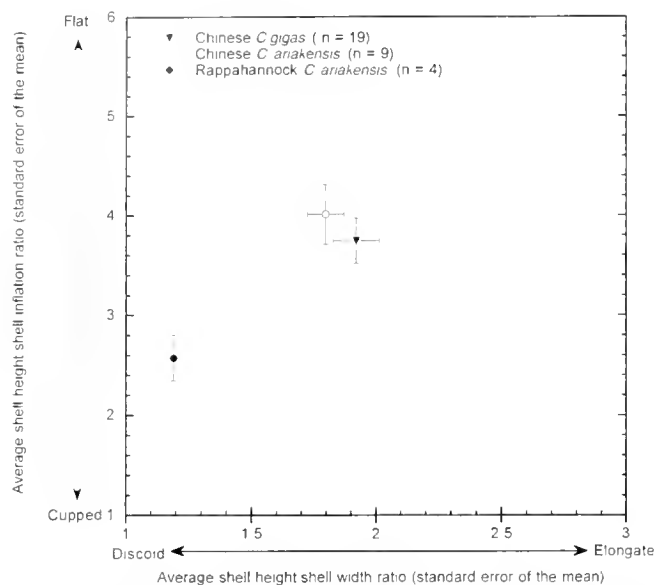


Figure 4. Average shell height (SH): shell width (SW) ratios (with standard error of the mean) in relation to average shell height: shell inflation (SI) ratios (with standard error of the mean) for wild Chinese *C. ariakensis* and *C. gigas* and triploid Rappahannock *C. ariakensis*. Ratios of SH:SW and SH:SI for Rappahannock *C. ariakensis* are significantly less than these ratios for Chinese *C. ariakensis* (ANOVAS,  $P < 0.05$ ). Ratios of SH:SW and SH:SI are similar for both Chinese species (ANOVAS,  $P > 0.05$ ).

Oysters have the potential to live in excess of 10 y in the absence of disease pressure, environmental degradation or human fishing pressure (Richardson et al. 1993a, 1993b). Evolutionarily life history has evolved to ensure success of the species over the course of its lifetime; that is, each individual only has to reproduce successfully once to maintain the population. Recruitment in wild populations should not necessarily be expected every year given

TABLE 2.

Observed average shell height (SH) at age (standard error of the mean) and the observed range of shell heights at age for populations of Chinese *C. gigas* (CHCg), Chinese *C. ariakensis* (CHCa), and Rappahannock *C. ariakensis* (RACa) examined in this study. Measurements were made from internal growth lines within the left valve of each individual. SE = standard error of the mean.

Population	Age	<i>n</i>	Average SH (mm, SE) at Age	Range of Observed SH at Age (mm)
CHCg	1	19	39.31 (2.12)	27–55
	2	17	99.0 (3.78)	68–130
	3	5	123.0 (6.44)	105–145
	4	1	155 (NA)	NA
CHCa	1	9	42.7 (3.3)	27–57
	2	9	101.42 (6.05)	75–134
	3	6	136.83 (4.74)	120–152
	4	4	167.25 (5.23)	155–179
RACa	5	4	191.25 (8.14)	170–204
	1	4	43.38 (3.54)	35–50
	2	4	102.37 (5.85)	95–120
	3	4	136.48 (5.54)	125–150
	4	4	156.20 (5.91)	140–165

NA = Not applicable.

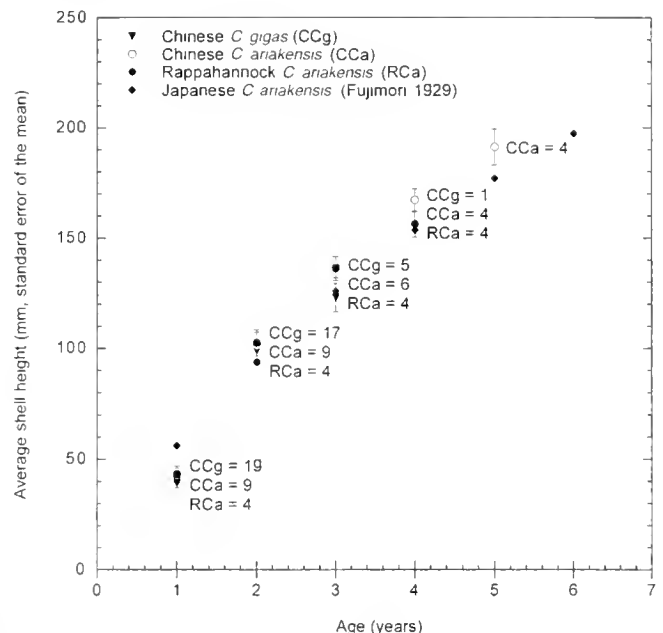


Figure 5. Average shell height (mm, standard error of the mean) at age for wild Chinese *Crassostrea ariakensis* and *C. gigas* and triploid *C. ariakensis* from the Rappahannock River, Virginia, United States in relation to reported shell-height at age for *C. ariakensis* in Ariake Bay, Japan (Fujimori 1929 reported by Cahn 1950). *n* values for each species at age are presented to the right of each data point for Chinese and Rappahannock River animals.

the potential for interannual variability in recruitment to estuarine and marine benthic habitats (Loosanoff 1966, Powell & Cummins 1985). The observed age distribution within wild collections (11 2-y-old *C. gigas* out of 19 total, four 5-y-old *C. ariakensis* out of 9 total) indicates that we are seeing cohorts. Size variation within

TABLE 3.

Summary of published shell-heights at age for *C. ariakensis* and *C. gigas*.

<i>C. ariakensis</i>			<i>C. gigas</i>		
Age	SH (mm)	Location	Age	SH (mm)	Location
1	55	Japan <sup>1</sup>	1	20.1 (SD 6.1)	Portugal <sup>3</sup>
	45	Washington, US <sup>2</sup>		24.4	Mexico <sup>4</sup>
	45–50	Oregon, US <sup>2</sup>		30	Washington, US <sup>2</sup>
	30–60	California, US <sup>2</sup>		40–100	Oregon, US <sup>2</sup>
2	97	Japan <sup>1</sup>		50	California, US <sup>2</sup>
	100	Washington, US <sup>2</sup>	2	60–70	Portugal <sup>3</sup>
	65–90	Oregon, US <sup>2</sup>		65.6	Mexico <sup>4</sup>
	110	California, US <sup>2</sup>		110	Washington, US <sup>2</sup>
3	124	Japan <sup>1</sup>		70–90	Oregon, US <sup>2</sup>
4	152	Japan <sup>1</sup>		145	California, US <sup>2</sup>
5	179	Japan <sup>1</sup>			
6	197	Japan <sup>1</sup>			

<sup>1</sup> Ariake Bay, Japan. Fujimori 1929 from Cahn 1950.

<sup>2</sup> Puget Sound, Washington, US; Yaquina and Coos Bay, Oregon, US; Tomales Bay, California, US. Langdon and Robinson 1996.

<sup>3</sup> Rio de Aveiro and Mondego River estuary, Portugal. Almeida et al. 1997.

<sup>4</sup> Bahia de La Paz, Mexico. Arizpe 1996.



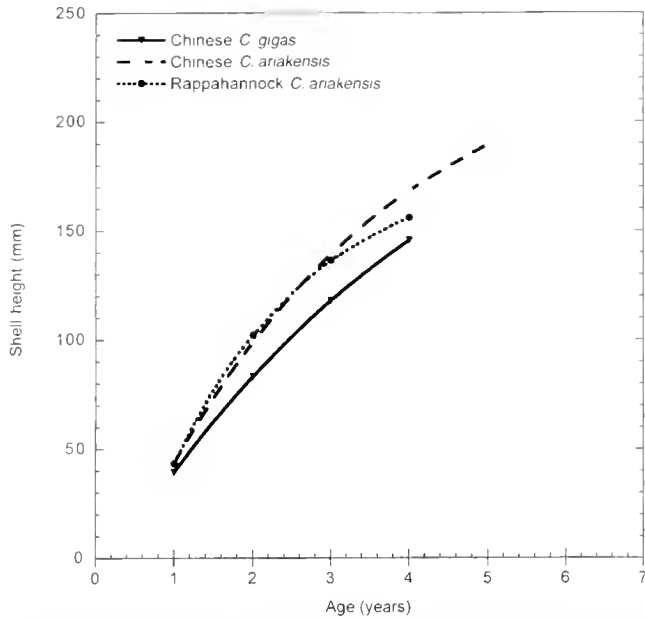


Figure 6. Fitted Von Bertalanffy growth curves for wild Chinese *Crassostrea ariakensis* and *C. gigas* and triploid *C. ariakensis* from the Rappahannock River, Virginia, United States.

a cohort may reflect time of settlement (early vs. late, see earlier), position in reef structure, or local conditions in the microhabitat in which the animal settled.

Comparisons of shell height at age and fitted growth curves present one element of comparison between oyster species within and across sites but these comparisons do not address the difference in inflation rates/cupping observed between the Rappahannock triploid *C. ariakensis* and the wild oysters of both species. The discoid, cupped Rappahannock oysters were larger in all dimensions than the Chinese oysters. When the four triploid Rappahannock *C. ariakensis* were shucked in May 2004, the meat fully filled the shell cavity with an average meat wet weight of 117.5 g (standard error of the mean, SE = 7.43 g) and average dry weight of 27.01 g (SE = 2.24 g). J. Harding, unpublished data). This effective increase in shell volume has a direct correlation to the biomass of the animal and, in diploid individuals, fecundity. In the absence of tissue weights (wet or dry) for the diploid Chinese oysters, direct comparisons of biomass within a species (triploid vs. diploid), site (*C. ariakensis* versus *C. gigas*) or within species across sites are impossible, although it is probable that biomass trends for sites and species follow the observed trends in external morphology. In the absence of detailed habitat or ecological in-

TABLE 4.

Von Bertalanffy growth model coefficients (standard error), coefficient of determination ( $R^2$ ), and mean square of residual values for populations of Chinese *C. ariakensis* (CHCa), Chinese *C. gigas* (CHCg), and Rappahannock *C. ariakensis* (RACa). Residual mean square values are from the linear regression of observed versus predicted shell height (see text).

Population	Age/n	SHmax	k	$t_0$	$R^2$	Residual Mean Square
CHCg	1/19, 2/17,	158.6	0.68	0.58	0.89	383.22
	3/5, 4/1	(20.25)	(0.20)	(0.08)		
CHCa	1/9, 2/9,	244.0	0.33	0.40	0.94	125.33
	3/6, 4/4,	(30.41)	(0.08)	(0.13)		
	5/4					
RACa	1–4/4	183.0	0.55	0.51	0.96	92.19
		(19.14)	(0.15)	(0.51)		

formation for either collection site (e.g., annual temperature and salinity profiles, sediment characterization, population density) it is impossible to attribute observed differences in morphology to genetic (triploid vs. diploid), habitat, or ecological factors including competition for space or resources. Application of these growth trajectories to other oyster populations and habitats must include consideration of genetics and individual morphology (biomass) as well as ambient seasonal salinity and water temperature profiles for the habitats of interest.

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## UNDERSTANDING THE SUCCESS AND FAILURE OF OYSTER POPULATIONS: CLIMATIC CYCLES AND *PERKINSUS MARINUS*

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**ABSTRACT** *Perkinsus* (= *Dermocystidium*) *marinus* is a major cause of mortality in eastern oysters, *Crassostrea virginica*. Because initiation of infection and progression of disease are favored by high temperature and high salinity, we hypothesized that climatic cycles influence cycles of disease. Analyses of a 10-y time series of disease prevalence and intensity, chlorophyll *a*, suspended sediments, water temperature and salinity from a Louisiana site, using a wavelet technique, show a teleconnection between the El Niño–Southern Oscillation (ENSO) and oyster disease in the northern Gulf of Mexico. Salinity increases precede increased disease prevalence by several months. The changes in salinity that trigger changes in disease prevalence and intensity are strongly driven by ENSO events. Interannual variation is important in the initiation and intensification of disease, and salinity is the primary driving factor. The patterns in the environmental and disease time series suggest that epizootics can be initiated within 6 mo of a La Niña event, which produces increased water temperature and salinity. This relationship suggests an approach for predicting epizootics of *P. marinus* from climate models, which in turn can inform the management of oyster populations.

**KEY WORDS:** *Crassostrea virginica*, *Perkinsus marinus*, climate, wavelet analysis, seston

### INTRODUCTION

A critical factor determining the success or failure of oyster populations is the initiation and progression of *Perkinsus* (= *Dermocystidium*) *marinus*, the causative agent of “Dermo” disease in eastern oysters (*Crassostrea virginica*). As a major cause of oyster mortality, *P. marinus* can control oyster population dynamics (Ray 1954, Quick & Mackin 1971), and hence the economic viability of the oyster-growing enterprise. Disease initiation and progression are favored by high temperature and high salinity (see Soniat 1996), and thus climatic cycles likely influence cycles of disease (Powell et al. 1992, 1996; Kim & Powell 1998).

Of particular importance to the oyster industry along the Gulf of Mexico is a teleconnection to the El Niño southern-oscillation (ENSO). El Niño is a disruption of the oceanic-atmospheric system of the tropical Pacific that occurs when the trade winds, which blow from east to west, relax allowing warm water to accumulate along the equator producing a reduction in upwelling of cold water in the eastern Pacific Ocean. One result of El Niño conditions is changes in the amount and pattern of rainfall, with the Gulf States being typically cooler and wetter during El Niño years. In contrast, La Niña conditions are associated with stronger trade winds and increased upwelling in the eastern tropical Pacific. During La Niña events, the Gulf States are warmer and drier (Ropelewski & Halpert 1986, Philander 1989). Kim and Powell (1998) suggested that infection intensity of *P. marinus* along the Gulf of Mexico follows the ENSO cycle; lower disease levels are associated with cooler, wetter El Niño years, whereas higher disease levels are associated with the warmer, drier La Niña years.

A major impediment to linking ENSO and Dermo disease along the Gulf of Mexico is the lack of appropriate long-term data sets. In the first study of this series (Soniat et al. 1998) we investigated the importance of what to measure and when to measure it. This study presents analyses of a 10-y time series of disease prevalence and intensity, components of the seston, and water tempera-

ture and salinity from a Louisiana site. Through the technique of wavelet analysis these time series are examined to determine interannual interactions among ENSO, water temperature and salinity, chlorophyll, suspended sediments and oyster disease. The results of the analyses are then used to determine the effects of interannual variation in climate on the initiation and progression of oyster disease.

### MATERIALS AND METHODS

#### *Environmental and Oyster Samples*

Environmental variables were measured weekly and oysters were sampled monthly from February 2, 1992 to February 14, 2002 at a single semiprotected reef (29°11'11"N, 90°39'56"W, GPS) that is located between Bayou Petit Calliou and a small marsh island in Bay Tambour, Terrebonne Parish, Louisiana. Water depth at the site varied from 0.3–0.6 m.

Temperature (T) and salinity (S) at the study site were measured at weekly intervals; T was recorded to the nearest 0.1°C using a mercury thermometer, whereas S was measured to the nearest 0.5 ppt using a refractometer (Behrens 1965). Seston variables that included chlorophyll *a* (CHL), total suspended solids (TSS), particulate organic matter (POM) and particulate inorganic matter (PIM), were also measured weekly. Water was sampled from 0.3 m above the reef with a hand-operated peristaltic pump. For the determination of CHL, 25 mL of water was filtered through 25 mm Whatman GF/F glass fiber filters, extracted in 5 mL of 60:40 v/v acetone:dimethyl sulfoxide, and read on a Turner Designs Model 10 fluorometer (Shoef & Lium 1976). For the determination of TSS, POM and PIM, 100–250 mL of water (depending upon Secchi depth) was filtered through 47 mm Gelman A/E glass fiber filters. The filters and filtrate were dried for 1 h at 103°C to obtain TSS and then ashed at 550°C for 15 min to determine PIM (American Public Health Association 1971).

Oysters were collected by tonging. A subsample of 10 commercial size oysters (>75 mm) were culled, cleaned of epifauna and measured (anterior to posterior length) to the nearest mm. A

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small piece of mantle tissue (about 4 mm<sup>2</sup>) was used to assay *Perkinsus marinus* (Ray 1966). Level of infection was scored using Mackin's 0-to-5 scale as modified by Craig et al. (1989). Percent infection (PI) and infection intensity (II) were determined, where

$$II = \frac{\text{sum of Mackin's disease code numbers}}{\text{number of infected oysters}}$$

#### Climate Data

The climate signature was represented by the El Niño 3.4 sea surface temperature index (EN) for the tropical-Pacific rectangle (5°N to 5°S, 170°W to 120°W) taken from the National Oceanic and Atmospheric Administration National Center for Environmental Prediction web site ([www.cpc.ncep.noaa.gov/indices/Readme.index.htm](http://www.cpc.ncep.noaa.gov/indices/Readme.index.htm)). High values of this index coincide with El Niño conditions. The EN is available at monthly intervals and the value was assumed to apply to the middle of the month. The index was linearly interpolated to the times of the weekly observations.

#### Wavelet Analysis

The time series technique of wavelet analysis (Torrence & Compo 1998) was used with the disease and environmental data sets to investigate interannual relationships among ENSO, seston, water temperature and salinity, and oyster disease. Wavelet analysis has been applied to the study of tropical convection (Weng & Lau 1994), ENSO (Gu & Philander 1995), atmospheric cold fronts and temperature fluctuations (Gamage & Blumen 1993, Baliunas et al. 1997), ocean waves and turbulent flows (Farge 1992, Meyers et al. 1993, Liu 1994), tidal phenomena (Flinchem & Jay 2000), phytoplankton distribution (Machu et al. 1999), and symphonic music (Strang 1994). To our knowledge, this is the first application of wavelet analysis the study of shellfish.

Wavelet analysis resolves localized variations in the strength of a signal (i.e., the wave) within a time series. With this approach, the original time series is decomposed into a time-frequency space, which allows the dominant components (i.e., the wavelets) that make up the wave to be identified. For example, in a wavelet study, water height might be decomposed into semidiurnal/diurnal,

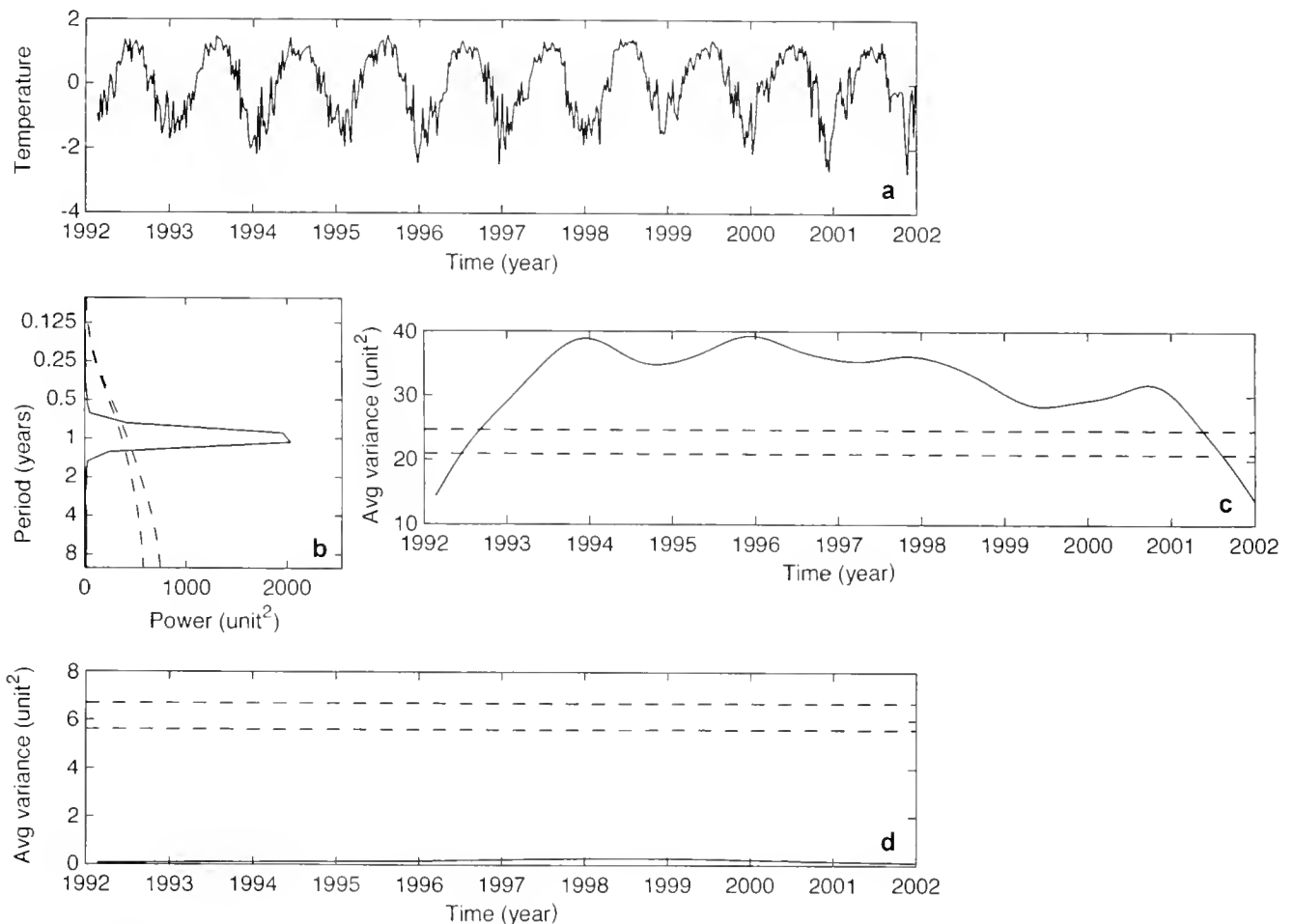


Figure 1. Wavelet analysis of temperature (T). a. Variation in the T time series. b. Power and periodicity of the T signal. The location of the solid line shows the periodicity of the signal, whereas its height represents the power of the signal. The dashed line to the left is the  $P < 0.10$  significance level and the dashed line to the right is the  $P < 0.05$  significance level. c. Variance averaged over an annual cycle, note that the annual T variation is highly significant over the time period. (The lower dashed line is the  $P < 0.10$  significance level and the upper dashed line is the  $P < 0.05$  significance level.) d. Variance averaged over 2–5 y, a range that encompasses the ENSO signal. Note that T is not significant when the variance is averaged over 2–5 y. The nonsignificant values at the beginning and end of the time series arise because the wavelet analysis assumes a periodic time series in which the values at each end match. For the time series used in this analysis, this assumption is violated. The peak that is produced by the mismatch results in artificial small scale variability that causes the lack of significance at the ends of the time series.

fortnightly, and seasonal (wind field) components (Flinchem & Jay 2000). Mathematical details of the wavelet technique are given in Daubechies (1992) and Torrence and Compo (1998).

A wavelet analysis was done for each variable (T, S, PI, II, EN, CHL, PIM) using two different mother wavelets (Paul & Morlet wavelets), which provide an approach for filtering the original time series. The Morlet wavelets give good frequency resolution but smear the dominant signals in the time domain. The Paul wavelets provide good time resolution but smear the signals in the frequency domain. Comparisons of the results from the two approaches showed that the Morlet wavelet provides adequate time resolution and superior frequency resolution over the results obtained with the Paul wavelet. Hence, only the results from the Morlet wavelet analyses are reported. Cross-wavelet analyses (CWA) were done on selected combinations of variables (T  $\times$  CHL, T  $\times$  S, T  $\times$  PI, T  $\times$  II, T  $\times$  EN, S  $\times$  PI, S  $\times$  II, S  $\times$  EN, PI  $\times$  II, PI  $\times$  EN, II  $\times$  EN) to determine correlations between different environmental and biologic processes, which in turn can suggest causative linkages.

## RESULTS

Over the time of the field study, water temperature varied from 4.8°C to 32.2°C with a 10-y mean of 22.5°C (Fig. 1a). The power and periodicity of the temperature signal show a single significant periodicity centered at 1 y but extending across periods of 0.5–2 y. The periodicity is significant throughout the time series (Fig. 1c). Averaging the variance over 2–5 y, which includes the frequency of the occurrence of El Niño events, shows no significant signal in the temperature time series (Fig. 1d). This result indicates that water temperature at the study site is not significantly affected by El Niño conditions.

Analysis of the Niño 3.4 anomaly shows a strong El Niño signal in late 1997 and early 1998 (Fig. 2a). A moderate and extended El Niño occurred during 1992 to 1995. The energy spectrum (Fig. 2b) shows significance at periods of about 2.5–5 y. Analysis of the 2–5 y portion of the energy spectrum over the time series shows that this component is significant over essentially the entire time (Fig. 2c).

The chlorophyll *a* measured at the study site, varied from 1.2–55.4  $\mu\text{g/L}$  with a 10-y mean of 11.4  $\mu\text{g/L}$  (Fig. 3a). Chlorophyll has a strong and significant peak in the power spectrum at 1 y (Fig. 3b), similar to what was obtained for temperature. Cross wavelet analysis (CWA) between temperature and chlorophyll (Fig. 4a) shows significant coherence at periods of 1 y or less (Fig. 4b), which indicates that the two time series are significantly correlated at this time scale. Moreover, the 1-y phase curve (Fig. 4c) falls at about zero degrees indicating that temperature and chlorophyll are in phase at periods of 1-y; high temperature coincides with high chlorophyll. The temperature and chlorophyll time series also show coherence, although weaker, at 4 y, suggesting some multi-year coherence in the time series. The 4-y periodicity is not well developed in the analysis of the individual time series (Figs. 1c and 3c), confirming that the multiyear structure in the cross correlation is not a dominant signal.

Over the 10 y sampled, salinity ranged from 0.5–29.5 ppt, with a grand mean of 15.2 ppt (Fig. 5a). The power spectrum shows a significant peak in salinity at 4 y (Fig. 5b). In contrast to temperature, no strong annual periodicity exists in the salinity signal. The power spectrum shows that the 4–6 y portion is significant for most of the time series (Fig. 5c). Clearly, temperature and salinity are not operating in phase seasonally. In contrast, a salinity versus EN CWA (Fig. 6a) shows a barely significant peak at 2 y and a more significant peak at 4 y (Fig. 6b). The 4-y peak coincides with both power spectra of the primary analysis (Figs. 2b and 5b). The

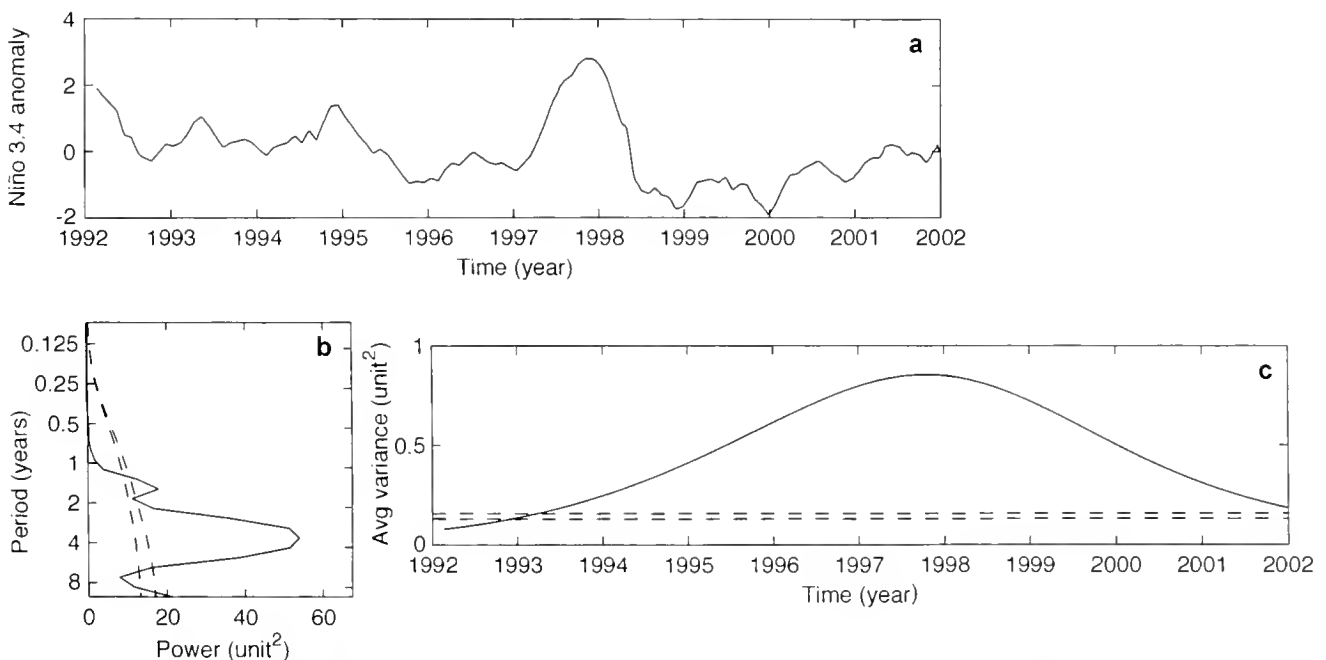


Figure 2. Wavelet analysis of the Niño 3.4 anomaly (EN). a. Variation in the EN time series. Note the strong EN signal during late 1997 and early 1998. b. Power and periodicity of the EN signal. Note the strong signal at a period of 4 y. c. Variance averaged over 2–5 y showing that EN is significant over essentially the entire time series.

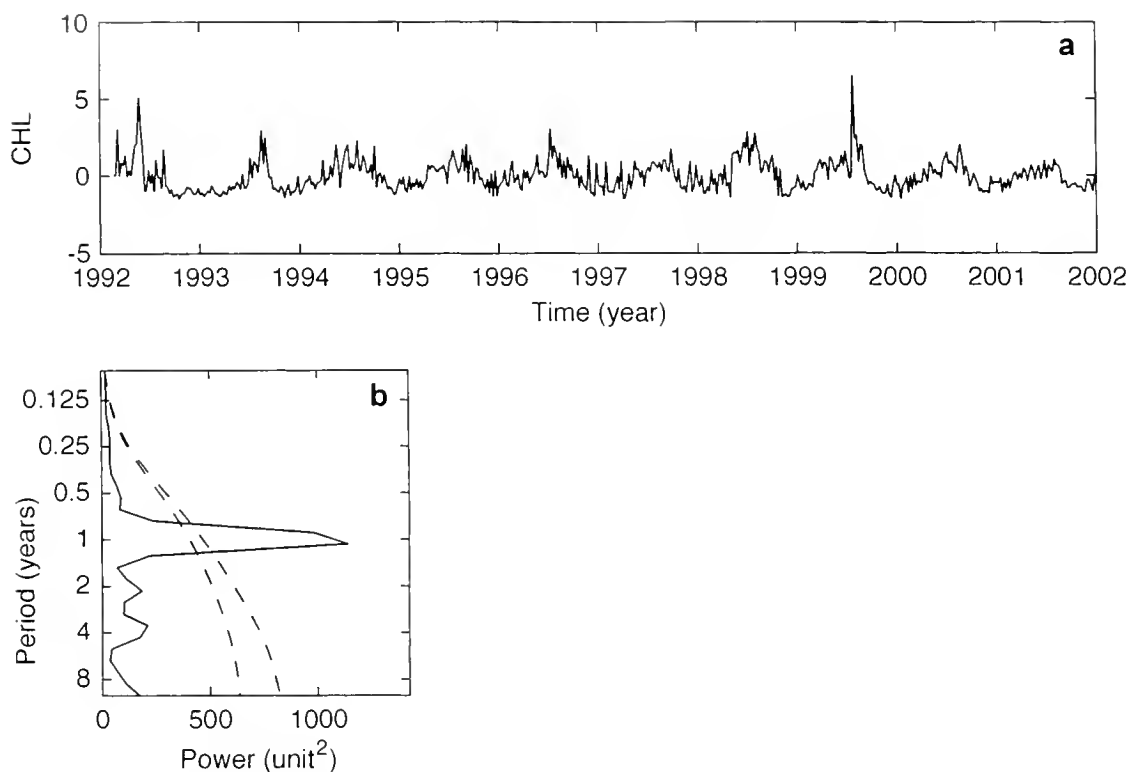


Figure 3. Wavelet analysis of chlorophyll *a* (CHL). a. Variation in the CHL time series. b. Power and periodicity of the CHL signal. Note the strong annual signal.

4-y periodicity of the two time series are roughly  $180^\circ$  out of phase (Fig. 6c). Thus, high salinity is associated with a low Niño 3.4 anomaly—the La Niña condition.

Particulate inorganic matter, which is a measure of resuspended sediment, varies from 4.0–531.33 mg/L with a grand mean of 50.2 mg/L (Fig. 7a). The power spectrum derived from this time series is not significant except at periods much less than 1 y (Fig. 7b).

and is not significant over the 2–5 y El Niño portion of the power spectrum (Fig. 7c).

Dermo percent infection (PI) varied from 0% to 100%, with an average of about 80% of the oysters being infected over the years sampled (Fig. 8a). The power spectrum obtained from this time series has peaks at 0.5 and 1.5 y (Fig. 8b), both of which are likely indicative of seasonal shifts in prevalence. The 0.5–2 y portion of

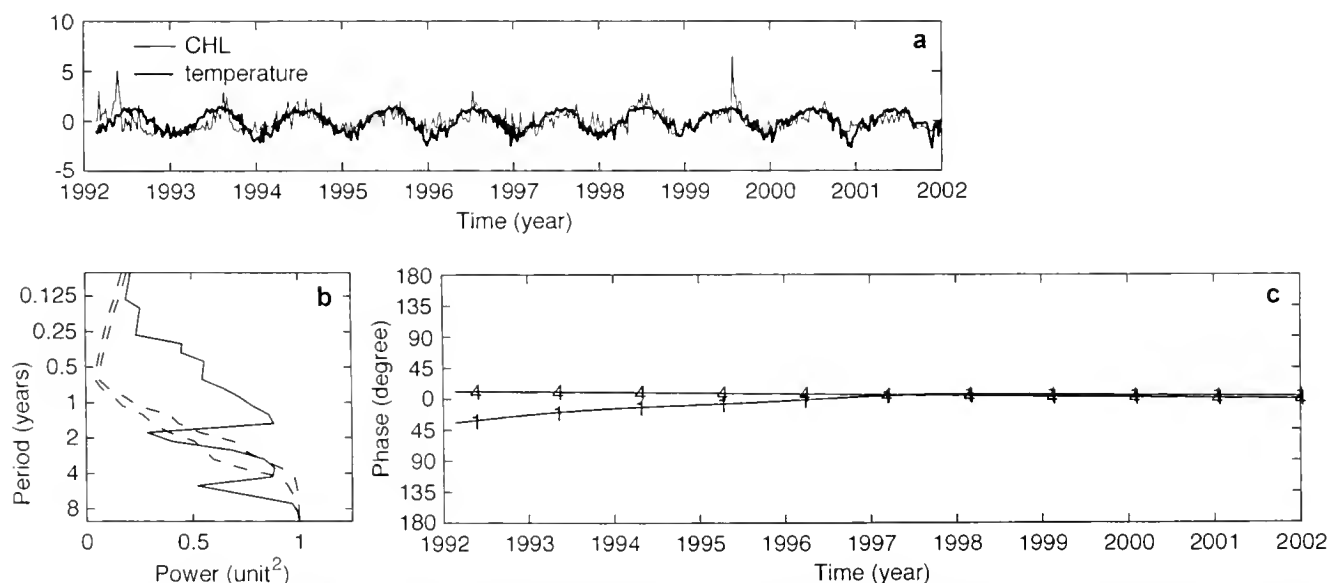


Figure 4. A Cross Wavelet Analysis (CWA) of chlorophyll (CHL) × temperature (T). a. Variation in the CHL and T time series. b. Power and periodicity of the CHL × T CWA. Note the strong signal at 1 y. c. Phase diagram. Note the phase of the 1-y periodicity is about 0 degrees, indicating that high T coincides with high CHL.

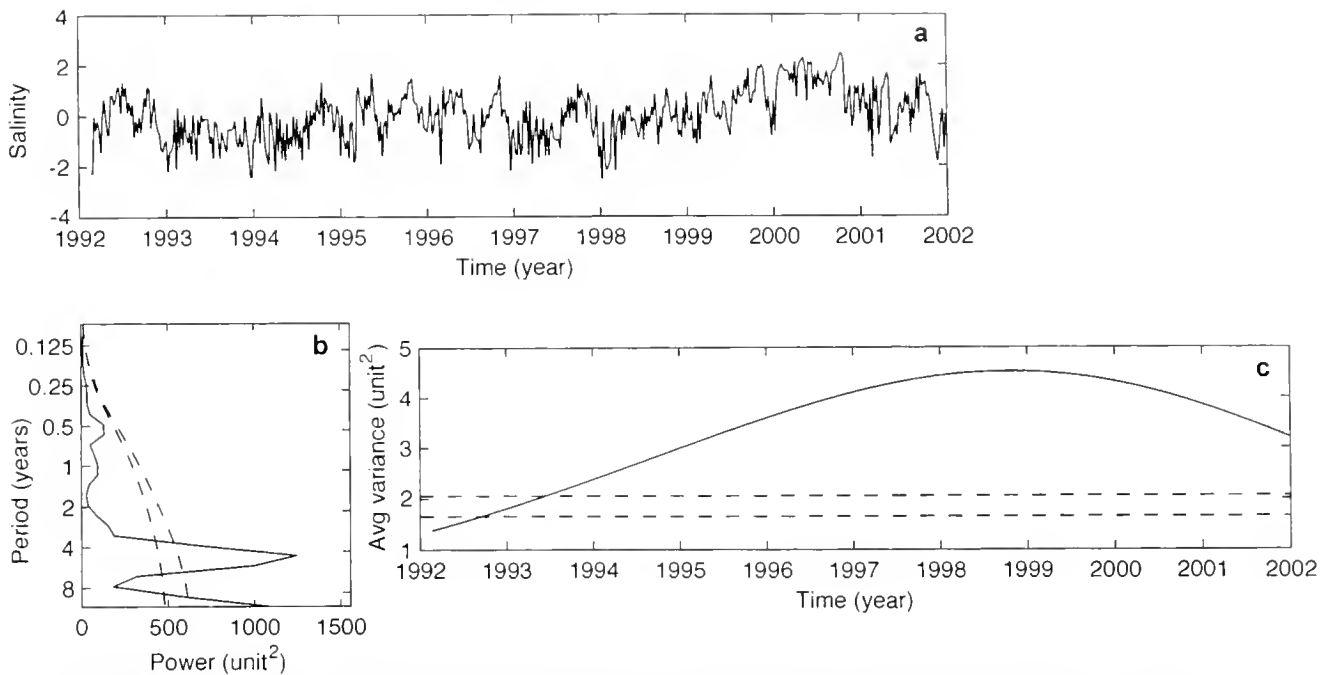


Figure 5. Wavelet analysis of salinity (S). a. Variation in the S time series. b. Power and periodicity of the S signal. Note the lack of an annual signal and the strong 4–6 y signal. c. Variance averaged over 4–6 y showing that S is significant over most of the time series.

the power spectrum is not significant over much of the time series (Fig. 8c), nor is the 2–5 y EN portion of the power spectrum significant (Fig. 8d).

The CWA between EN and PI, and salinity and PI show the 4-y peaks (Figs. 9b and 10b), which coincide with a peak, albeit non-significant, in the original PI spectrum (Fig. 8b). The 4-y trend in the EN  $\times$  PI CWA (Fig. 9c) is shifted by about  $140^\circ$ , so that high prevalence follows a low Niño 3.4 anomaly (a La-Niña high-salinity event) by about 1.5 y (phase/360  $\times$  period or  $140/360 \times 4$ ). The 4-y trend in salinity (Fig. 10c) is nearly in phase with the high

in prevalence. The phase shift is about  $-15^\circ$ , so high PI lags high S by about 1–2 mo.

Dermo infection intensity (II) ranged from 0.0–3.0, with an overall mean of 0.9 (Fig. 11a). The power spectrum from the Dermo II time series shows significant peaks at 0.5, 1, and 4 y (Fig. 11b). The shorter signals are subannual and annual periodicities arising from the normal seasonal progression of this disease. Averaging power in the 4–6 y period (the salinity signature, Fig. 5b) shows significance over much of the first half of the time series (Fig. 11c). Averaging power over 0.5–2 y (the temperature signal,

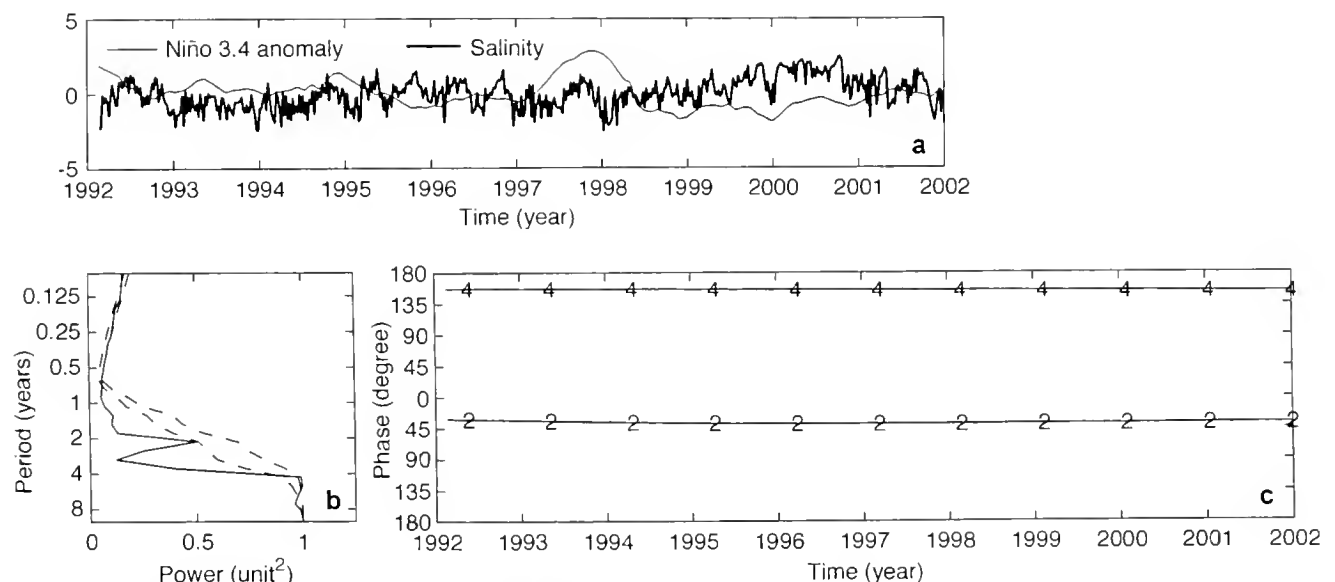


Figure 6. A CWA of the Niño 3.4 anomaly (EN) and salinity (S). a. Variation in the EN and S time series. b. Power and periodicity of the EN  $\times$  S CWA. Note the barely significant signal at 2 y and the more significant peak at 4 y. c. Phase diagram. Note that for the 4-y periodicity EN and S are nearly  $180^\circ$  out of phase.

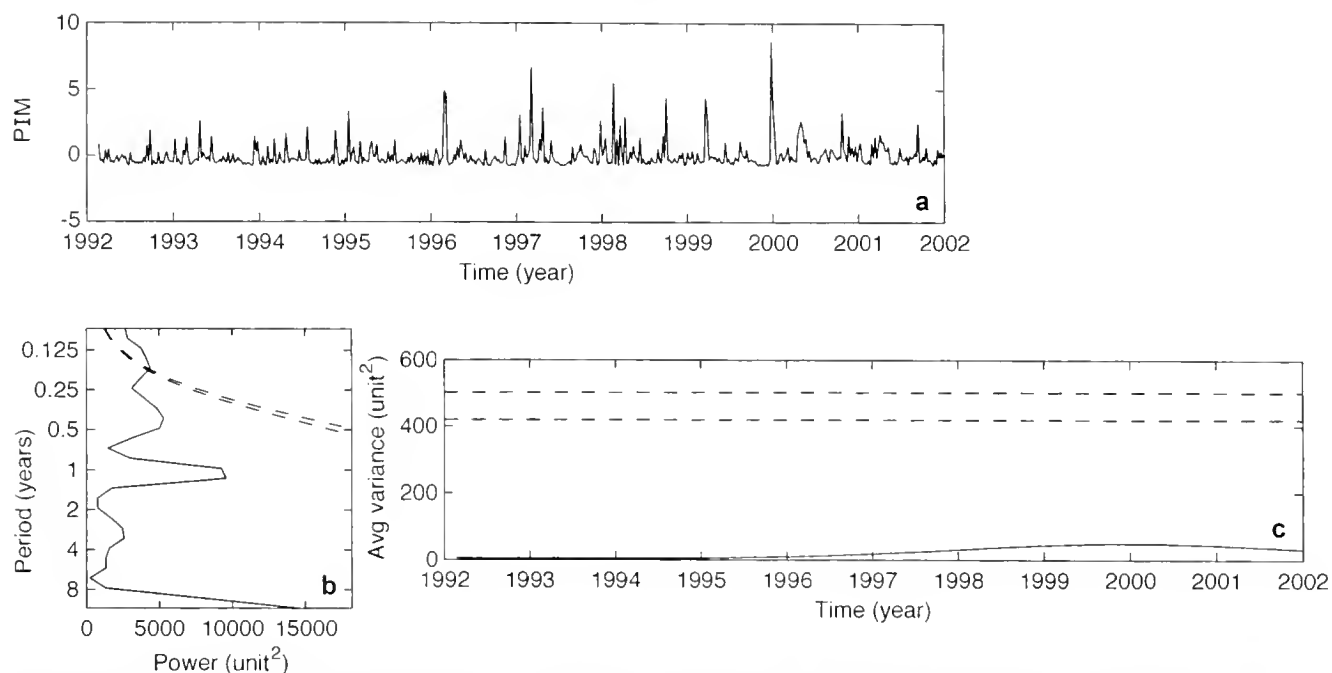


Figure 7. Wavelet analysis of particulate inorganic matter (PIM). a. Variation in the PIM time series. b. Power and periodicity of the PIM signal. Note the subannual signal. c. Variance averaged over 4–6 y showing that PIM is not significant over all of the time series.

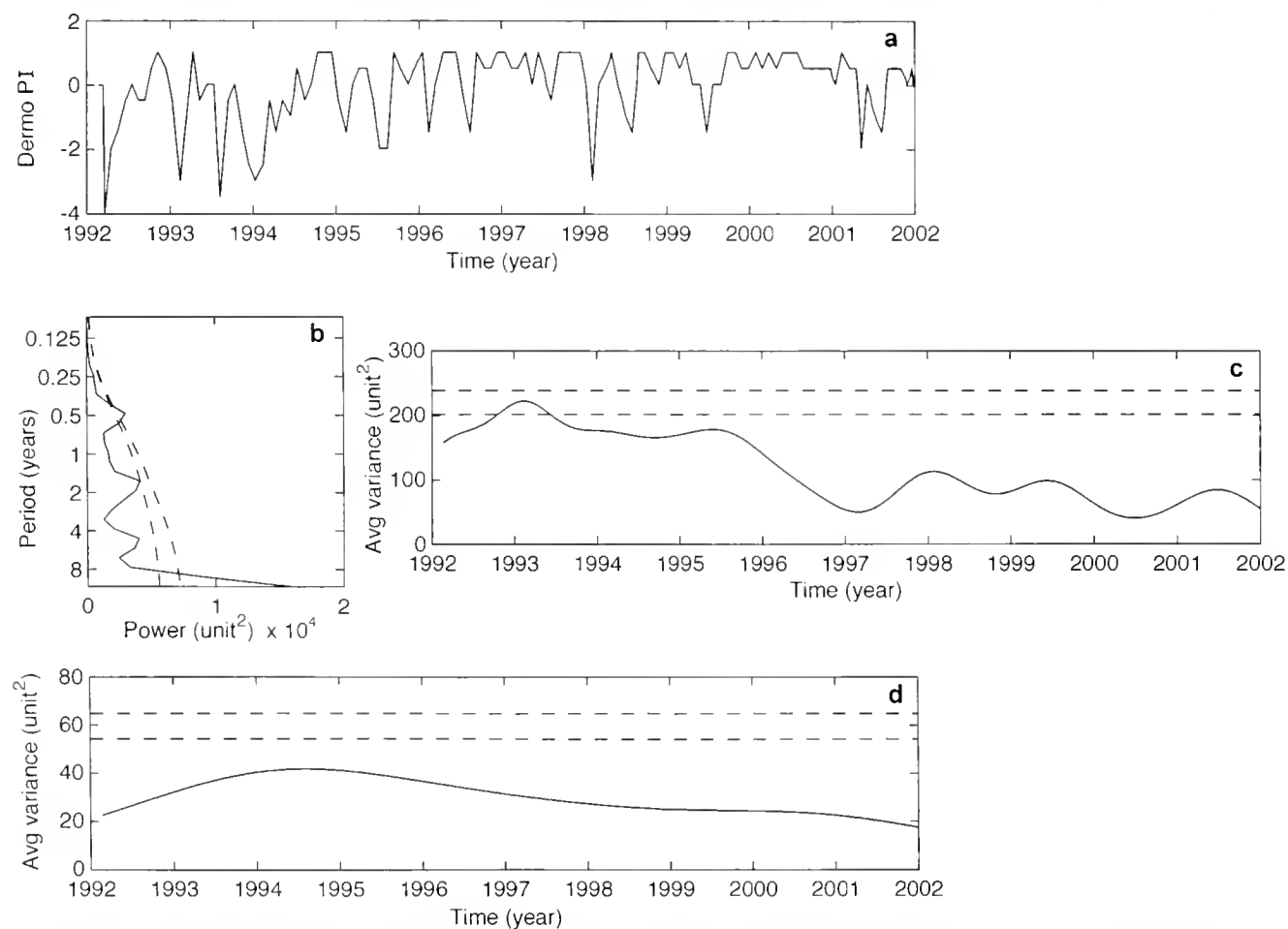
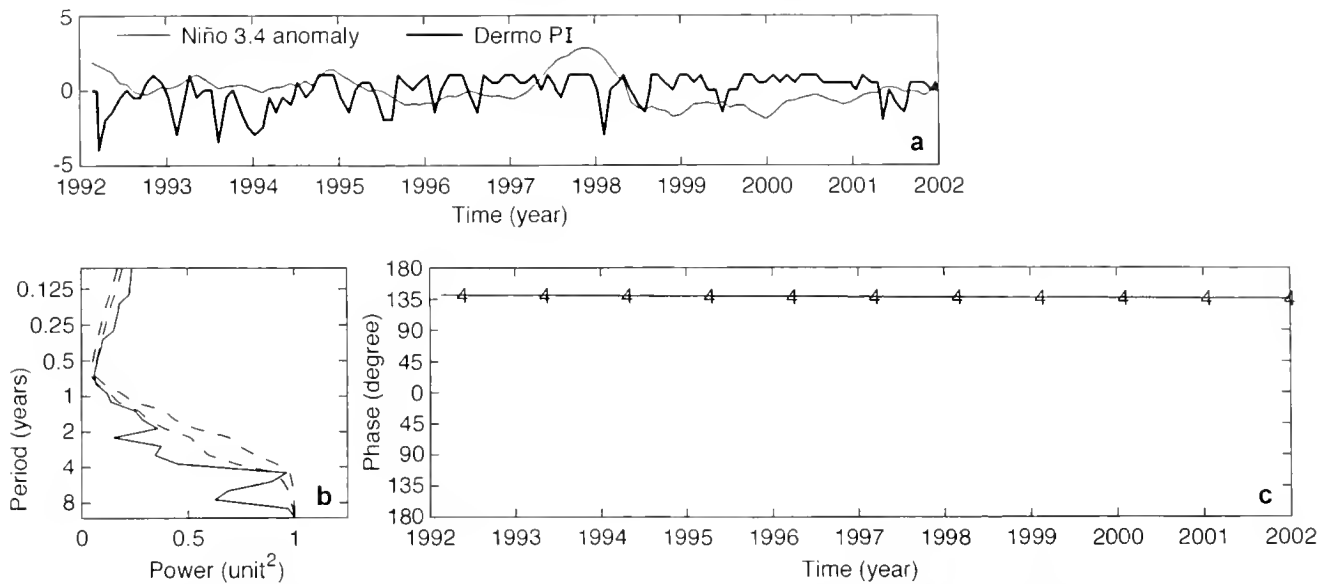


Figure 8. Wavelet analysis of Dermo percent infection (PI). a. Variation in the PI time series. b. Power and periodicity of the PI signal. Note the 0.5 and 1.5 y peaks. c. Variance averaged over 0.5–2 y showing that PI is not significant over much of the time series. d. Variance averaged over 2–5 y showing that PI is not significant over all of the time series.





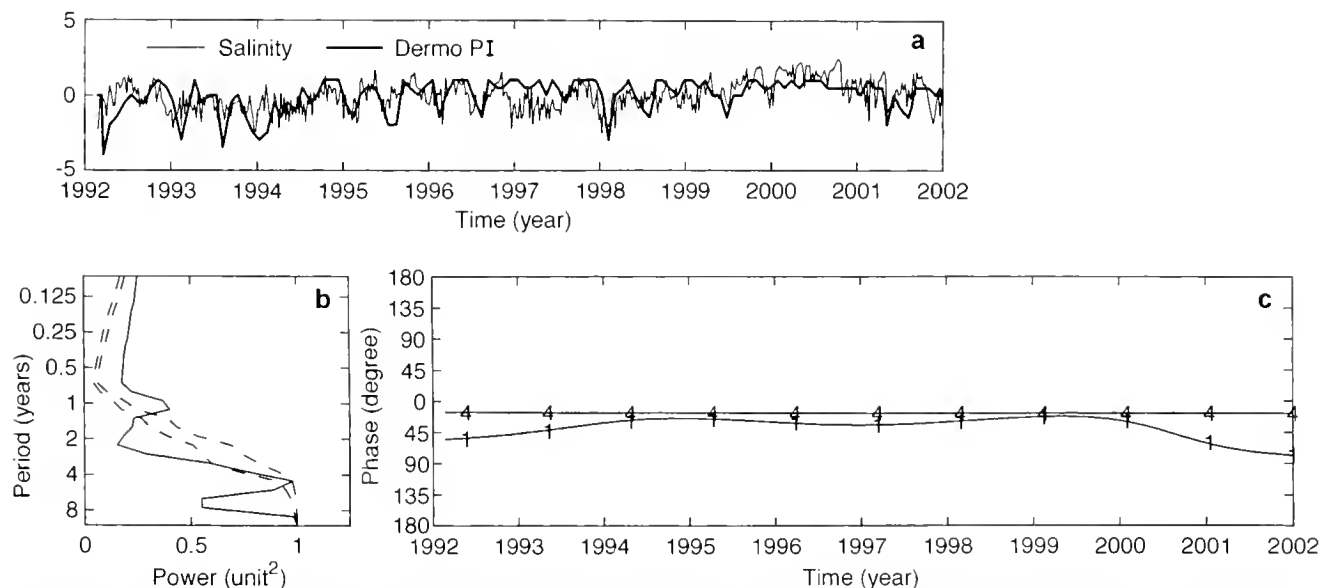
**Figure 9.** A CWA of the Niño 3.4 anomaly (EN) and Dermo PI. a. Variation in the EN and PI time series. b. Power and periodicity of the EN  $\times$  PI CWA. Note the barely significant signal at 2 y and the more significant peak at 4 y. c. Phase diagram. Note that EN and PI are about 140 degrees out of phase.

Fig. 1b) shows a strong significant peak in the center of the time series (Fig. 11d). Averaging power over 2–5 y (the EN signature, Fig. 2b) likewise shows a strong peak in the middle of the time series (Fig. 11e). This central portion of the time series coincides with the highest infection intensities (Fig. 11a), and also with the 1996 to 1997 La Niña event (Fig. 2a).

The CWA of Dermo II and salinity (Fig. 12) shows strong and significant coherence over short periods and 4 y (Fig. 12b). The 4-y periodicity in the Dermo II time series has a phase shift of  $-45$  degrees with respect to salinity—a phase shift of about 0.5 y. Thus, high salinity leads Dermo II by about 6 mo. The 1-y peak shows about a  $-30$ -degree shift (Fig. 12c). This is equivalent to a  $-30$  degrees/360 degrees  $\times$  1 y, or 0.08 y, or about a 1 mo phase shift.

Therefore, for the 1-y periodicity, an increase in salinity precedes Dermo II by about a month. A CWA of Dermo II and EN (Fig. 13) shows a strong peak at about 3–4 y (Fig. 13b) with a phase of  $+113^\circ$  (Fig. 13c), or about 1 y. That is, high Dermo II follows low Niño 3.4 anomaly values, the La Niña high-salinity event, by about 1 y.

The CWA of the Dermo PI and Dermo II time series (Fig. 14a) shows strong correlations at periods less than 1 y, at 2 y, and at 4 y (Fig. 14b). The 4-y peak has a phase shift of about  $-30$  degrees or 0.3 y (Fig. 14c). A rise in prevalence therefore leads a rise in intensity by about 4 mo. Note that high salinity precedes high prevalence by about 2 mo (Fig. 10c), and high salinity precedes intensity by 6 mo (Fig. 12c).



**Figure 10.** A CWA of the salinity (S) and Dermo PI. a. Variation in the S and PI time series. b. Power and periodicity of the S  $\times$  PI CWA. Note the significant signals at 2 and 4 y. c. Phase diagram. Note that the 4 y trend in S is nearly in phase with PI.

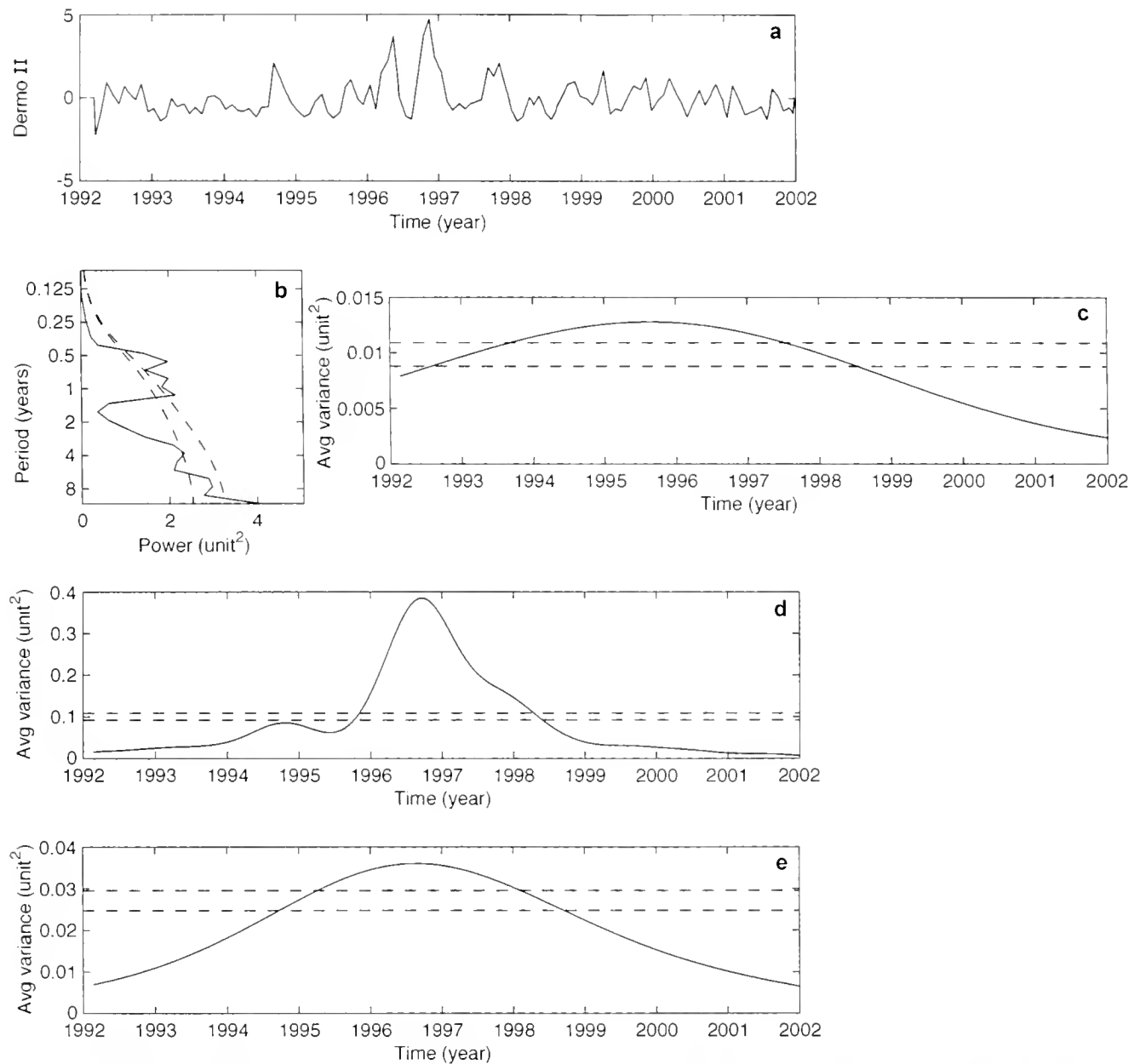


Figure 11. Wavelet analysis of Dermo infection intensity (II). a. Variation in the II time series. b. Power and periodicity of the II signal. Note the 0.5 and 1 and 4 y peaks. c. Variance averaged over 4–6 y showing that II is significant over much of the first half of the time series. d. Variance averaged over 0.5–2 y showing a strong significant peak in the center of the time series. e. Variance averaged over 2–5 y showing a significant peak in the center of the time series.

## DISCUSSION

For ENSO to exert an effect on disease levels of oysters in the northern Gulf of Mexico a number of necessary conditions must be met: (1) ENSO must have a teleconnection to weather along the Gulf, (2) weather must modify water temperature and/or salinity in local watersheds, and (3) interannual variation in ENSO must show synchrony with key environmental factors and disease levels. Ropelewski and Halpert (1986) reported an ENSO connection to the Gulf, in which El Niño years are cooler and wetter than typical years. Powell et al. (1992) found a high degree of concordance of yearly changes in disease between Gulf bays at spatial scales much

greater than 1,000 km, suggesting that PI and II are changing in response to broad shifts in weather patterns that likely directly affect local temperature and salinity (via rainfall and river runoff), and subsequently modulate PI and II.

The analyses of the time series from a site in southern Louisiana show periodicities at subannual, annual and interannual time scales. A high degree of synchrony in periodicities of measured variables with ENSO suggests interannual climatic variation as a driving force, whereas asynchrony suggests other temporal relationships such as subannual and annual control.

Particulate inorganic matter is the major component of the seston on which oysters feed. Excessive PIM can have deleterious

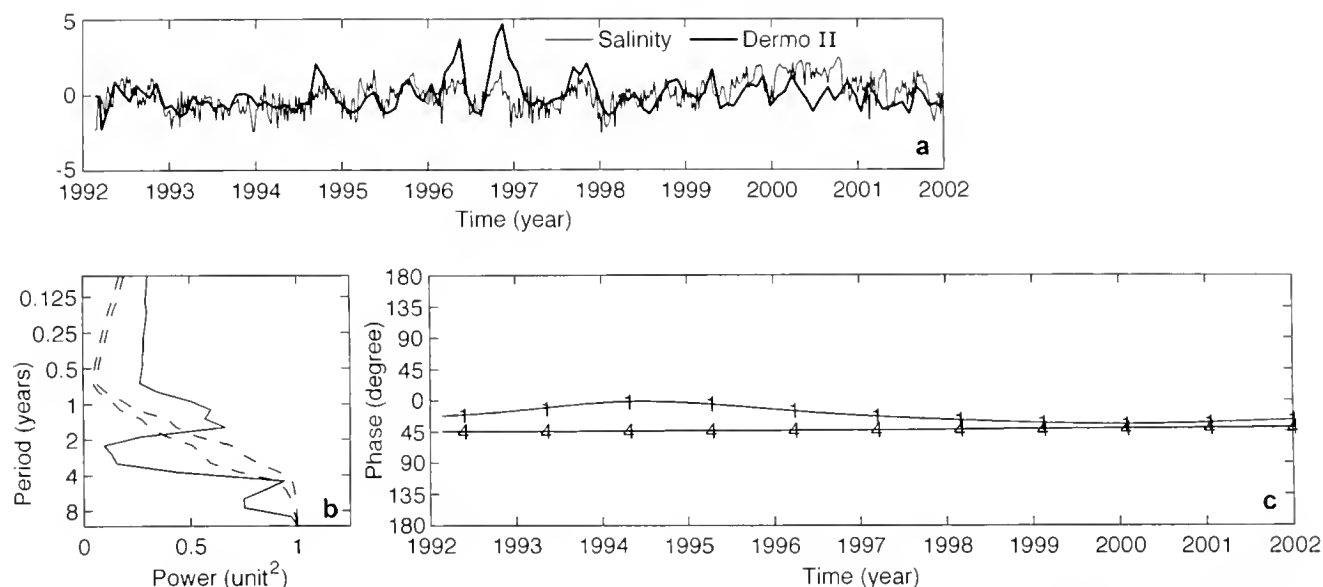


Figure 12. A CWA of the salinity (S) and Dermo II. a. Variation in the S and II time series. b. Power and periodicity of the  $S \times II$  CWA. Note the significant subannual, annual and 4 y signals. c. Phase diagram. Note that in the 4-y trend II lags S by about 45°.

effects on oysters by “diluting” its food supply. Chlorophyll *a* is a proxy for phytoplankton biomass and represents an important component of the oyster’s food supply (Soniat et al. 1998). Particulate load and food supply are important forcing functions controlling oyster growth and Dermo prevalence and intensity (Powell et al. 1996). These also mediate assimilation by altering filtration and ingestion rates and can thus determine the success or failure of oyster populations (Soniat et al. 1998). Sub-annual periodicity is clearly seen in the PIM time series, which is a record of the resuspension of bottom sediments caused by high-frequency wind events. Temperature and chlorophyll are coupled at annual time scales through temperature control of phytoplankton growth rate. Sediment resuspension, water temperature, and chlo-

rophyll have periodicities that are not in synchrony with the EN signal and are related only to a minor degree with changes in salinity.

Unlike temperature, salinity does not show an annual signal, but instead has a 4-y periodicity like that of the EN signal. Temperature and salinity are not in phase annually, but are in phase every 4 y. Seasonal high temperature coincides during these times with high salinity, established during the La Niña portion of the ENSO cycle: high temperature and high salinity are ideal conditions for the proliferation of disease. Disease II and PI have inter-annual periodicities that suggest a connection to the EN signal.

An increase in salinity is followed in several months by an increase in prevalence followed in several months by an increase

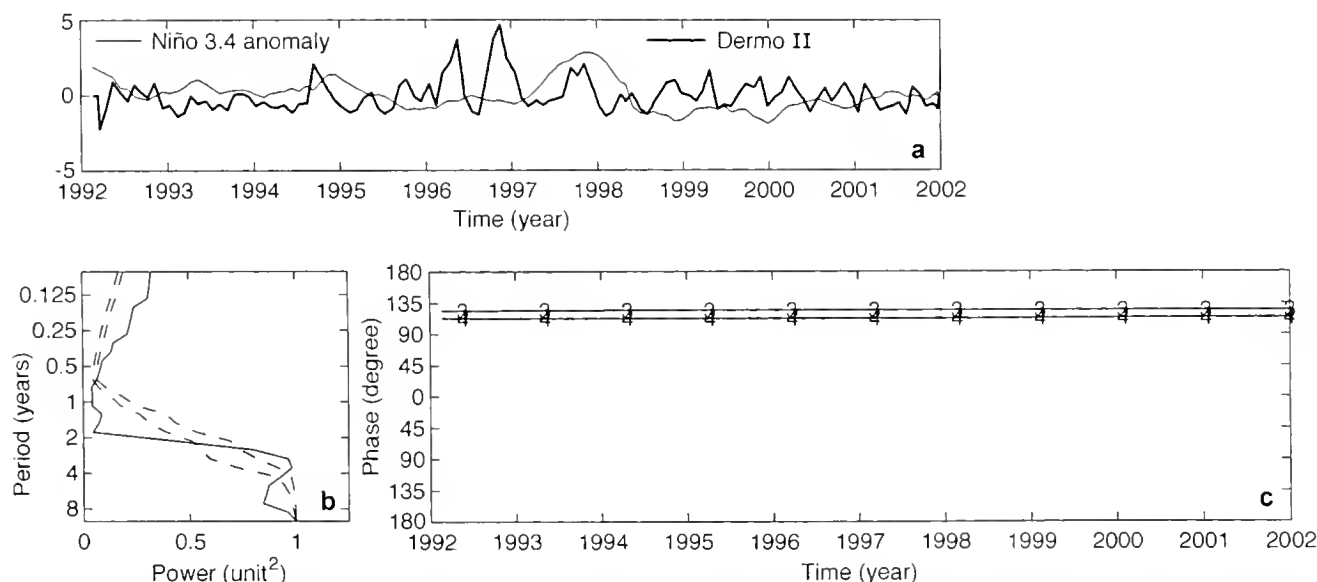


Figure 13. A CWA of the EN and Dermo II. a. Variation in the EN and II time series. b. Power and periodicity of the  $EN \times II$  CWA. Note the significant peak at 3-4 y. c. Phase diagram. Note that in the 4-y trend EN and II are about 113° out of phase.

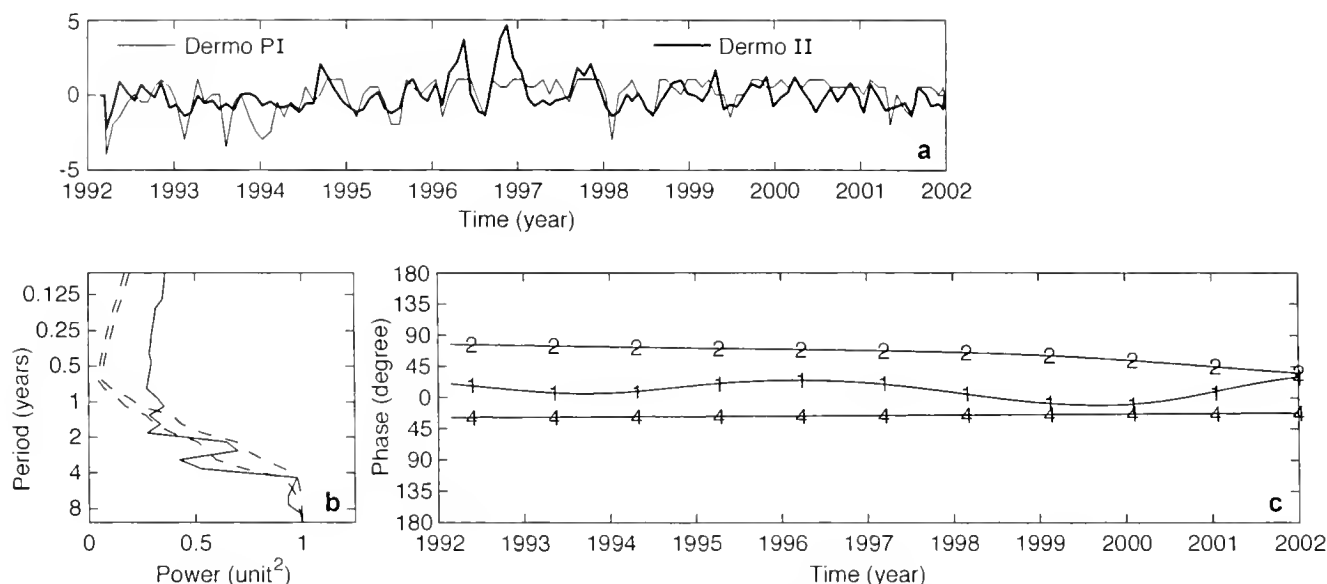


Figure 14. A CWA of the Dermo PI and Dermo II. a. Variation in the PI and II time series. b. Power and periodicity of the PI  $\times$  II CWA. Note the subannual/annual, 2 and 4-y peaks. c. Phase diagram. Note that in the 4-y trend PI and II are about  $\sim 30$  degrees out of phase.

in infection intensity, with salinity shifts strongly driven by ENSO events. Interannual variation is important in the initiation and intensification of disease, and salinity is the primary driving factor. This analysis of environmental and biologic time series from southern Louisiana show a teleconnection of ENSO to Dermo disease in the northern Gulf of Mexico. The correlations and phasing of signals in these time series suggest that epizootics of Dermo disease in oyster populations can be initiated within 6 mo of a La Niña event. This relationship makes it possible to potentially use results from climate models to predict epizootics of *P. marinus* in the Gulf Coast region. This provides a powerful approach for oyster management practices because it allows the possibility of an epizootic to be predicted several months in advance of the event,

which allows time for appropriate management practices to be implemented.

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## AN AFLP APPROACH TO IDENTIFY GENETIC MARKERS ASSOCIATED WITH RESISTANCE TO *VIBRIO VULNIFICUS* AND *PERKINSUS MARINUS* IN EASTERN OYSTERS

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**ABSTRACT** We conducted an arbitrary fragment length polymorphism (AFLP) analysis followed by tests of association to search for potential genetic markers associated with resistance to infection by *Vibrio vulnificus* and *Perkinsus marinus* in a sample of North Carolina oysters. We used 48 AFLP markers and found significant associations for two of these markers with the incidence of infection of *P. marinus* and two separate markers associated with the magnitude of infection of *V. vulnificus*. Further, there was evidence of epistatic interactions of genes affecting infection levels of both pathogens. This suggests existence of genes or groups of genes that are located on chromosome fragments close to the identified AFLP markers and that act directly or indirectly (through epistasis) to control the levels of infection by these microorganisms in oysters. The impact of the direct and epistatic effects of these unknown genes on infection level variability amounted to about 40% for both *P. marinus* and *V. vulnificus*. This study demonstrates the utility of the AFLP approach to identify genetic markers of pathogen/parasite resistance in eastern oysters. However, the identified AFLP markers are considered preliminary and suggestive only because of the limited sample size analyzed in this study. Further studies using this approach on a larger sample size are required to identify a set of robust molecular markers that would serve in a marker-supported breeding program designed to improve the quality of the oyster stock.

**KEY WORDS:** oysters, *Vibrio vulnificus*, *Perkinsus marinus*, AFLP markers, *Crassostrea virginica*

### INTRODUCTION

The eastern oyster *Crassostrea virginica* (Gmelin) is an economically and ecologically important bivalve species in eastern United States estuaries that serves as a host and a vector for a variety of prokaryotic and eukaryotic parasites. Among the parasites and pathogens that have the strongest impact on the oyster industry and wild oyster populations are a bacterium, *Vibrio vulnificus*, and a protozoan, *Perkinsus marinus*. *V. vulnificus* is a human pathogen common in estuarine waters around the world, and oysters are major vectors in the transfer of this bacterium to humans. Ingestion of raw or undercooked oysters containing this bacterium can result in illness and even death (Oliver & Kaper 2003). Although this bacterium does not negatively affect oyster survival and health, it causes significant losses to the oyster industry because of the required warning labels and associated negative publicity (Keithly & Diop 2001) and is a serious concern for public health in the United States, particularly in Gulf of Mexico states. In contrast, *P. marinus* is not harmful to humans but causes devastating effects on oyster populations. Epizootics of perkinsosis or dermo disease caused by this parasite are the main source of catastrophic mortalities in oysters that can wipe out up to 100% of the living stock in affected areas, threatening both aquacultured and wild oyster populations and leading to tremendous losses in the oyster industry.

Infection levels by *V. vulnificus* and *P. marinus* in oysters are controlled by various extrinsic factors such as environmental temperature and salinity (Crosby & Roberts 1990, O'Neill et al. 1992, Chu et al. 1993, Kaspar & Tamplin 1993, Bureson & Calvo 1996, Motes et al. 1998) but these factors cannot fully explain individual variation in infection levels in oyster populations. We showed that tissue loads of *V. vulnificus* and *P. marinus* vary greatly in oysters growing in the same habitat and exposed to similar environmental conditions, including levels of pathogens in the water (Sokolova et al. 2005). This variation is likely caused by the genetic differences

in resistance to infection; in fact resistance to *P. marinus* infection in *C. virginica* was earlier shown to have a significant genetic component and to depend on the host genotype and genotype-environment interactions (Oliver & Fisher 1999, Oliver et al. 2000). Overall, genetic variation in resistance to parasites and pathogens is well documented in mollusks and thus creates a potential basis for selection of parasite-resistant populations and strains (Grosholz 1994, Bushek & Allen 1996, Naciri-Graven et al. 1998, Langand & Morand 1998, Ataev & Coustau 1999, Knight et al. 1999, Wiehn et al. 2002).

Unfortunately, selective breeding applied to oysters has met with only limited success. Currently there are several oyster lines (e.g., Andrews DEBY and their descendants) that demonstrate some resistance to dermo disease (Ford et al. 1990, Calvo et al. 2003, S. K. Allen pers. comm.). Most of these lines, however, have not proven successful in growth locations outside of their sites of origin and may also suffer from the effects of inbreeding, which limits their use in aquaculture and oyster restoration programs. Further, no oyster strains with increased resistance to *V. vulnificus* infection are currently known. On the other hand, use of molecular genetic markers associated with resistance to parasites may strongly facilitate the current selective breeding programs and provide a noninvasive tool to detect resistant oysters from wild or cultured stock. Selection of parental stock from wild or cultured populations using molecular genetic markers would avoid the negative effects of inbreeding by diversifying the genetic background of breeders, whereas selecting for the desired parasite or pathogen resistance. However, this approach has never been applied to oyster stock, and no genetic markers associated with pathogen or parasite resistance are currently known in oysters. We therefore decided to test the feasibility of an arbitrary fragment length polymorphism (AFLP) approach to search for molecular genetic markers potentially associated with resistance to *P. marinus* and/or *V. vulnificus* infections. Here we report the details of an inexpensive technique that allows identification of such markers, and provide preliminary data on associations of AFLP marker loci with resistance of *C. virginica* to both *V. vulnificus* and *P. marinus*.

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## MATERIALS AND METHODS

### Animal Collection and Maintenance

Oysters were collected on June 15, 2004 from a subtidal habitat in Stump Sound, North Carolina. 50 adult oysters (55–155 mm valve height) were randomly collected from a small area (ca. 100 × 100 m) of homogenous soft-bottom habitat to ensure that all organisms used in this study were exposed to the same environmental conditions. Age of the oysters within this size range was 2–5 y as determined by the count of annual growth checks on their shells. Age determination by growth checks was verified by comparison with the growth checks on cultured oysters of known age grown subtidally in the nearby area of Stump Sound (courtesy of J. Swartzenberg, J & B Aquafood). Water temperature at the time of collection was 26°C and salinity was 31‰. Oysters were immediately placed on ice and transported to the University of North Carolina at Charlotte within 5 h of collection for further processing and analysis. Processing of oyster tissues was completed within 24 h of collection. During this time, oysters were kept on ice to prevent postharvest build-up of bacteria.

### Chemicals

AFLP adapters and preselective and selective primers were purchased from Integrated DNA Technologies (Coralville, IA). Restriction enzymes and T4 DNA ligases were purchased from New England Biolabs (Ipswich, MA). TaqPro Complete PCR mixture was purchased from Denville Scientific (South Plainfield, NJ). All other reagents were purchased from Fisher Scientific (Sewanee, GA) and Sigma (St. Louis, MO) and were of analytical grade or higher.

### Determination of *V. vulnificus* Loads and *P. marinus* Infection

Oysters were externally cleaned and opened with an alcohol-flamed oyster knife. The oyster contents were removed, weighed, and homogenized in sterile blender jars with an equal volume of sterile diluent (50% artificial sea water). Homogenates were diluted and plated for *V. vulnificus* using the cellobiose-polymyxin B-colistin (CPC) agar developed in our laboratory (Massad & Oliver 1987), which has been used by us and others for the primary isolation of *V. vulnificus* (Harwood et al. 2004, Oliver 2003). When colonies of appropriate color and morphology are selected, this medium has been shown to be 82% accurate in the isolation of *V. vulnificus* (Sun & Oliver 1995). Using these same criteria, Sloan et al. (1992) found 81% of the typical *V. vulnificus* colonies on CPC to be identified as this species.

Small samples of gill tissue (50–100 mg) were removed prior to homogenization and placed in DNA fixing solution for DNA extraction for AFLP analysis and PCR diagnostics of *P. marinus*. Remaining tissues were weighed to the nearest 0.01 g, and the maximum valve height was measured to the nearest 0.1 mm. For diagnostics of *P. marinus*, total DNA was isolated from 50–100 mg samples of gill tissue following an improved protocol for DNA isolation from mollusks developed by Sokolov (2000). This method allowed us to isolate total DNA, which in infected oysters contained DNA of *P. marinus* in addition to the host (oyster) DNA. Determination of *P. marinus* infection was performed using PCR with the following primers:

**Pmar-F:** 5' CAC TTG TAT TGT GAA GCA CCC 3'

**Pmar-R:** 5'GTG ACA TCT CCA AAT GAC C 3'

These primers are specific for *P. marinus* (Penna et al. 2001),

and do not cross-amplify with oyster DNA or other parasites. Optimized PCR conditions for *P. marinus* detection were as follows: 25 µL of reaction volume containing 1 × PCR buffer, 2 mM MgCl<sub>2</sub>, 100 µM dNTPs, 0.7 U of *Taq* polymerase, 150 ng of each *P. marinus* primer and 50–100 ng of template DNA was subjected to one denaturation cycle at 94°C for 5 min, 35 cycles at 94°C for 45 s, 55°C for 30 s and 72°C for 45 s and one final extension cycle at 72°C for 7 min. *P. marinus* DNA obtained from monocultures of this parasite (gift of Dr. G. Vasta) was used as a positive control. Amplified DNA fragments were resolved on ethidium bromide-stained 1.5% agarose gels and screened for the presence of a ca. 304 bp product characteristic of *P. marinus*, which indicated infection of the oyster with this parasite. This method is highly sensitive with detection limits of 0.1 pg DNA of *P. marinus*, corresponding to 1 protozoan cell g<sup>-1</sup> oyster tissue (Penna et al. 2001).

### AFLP Analysis

AFLP analysis was conducted according to an AFLP gene mapping protocol described elsewhere (Vos et al. 1995, Yu & Guo 2003, Li & Guo 2004). Isolated genomic DNA (~0.5–1 µg) was digested by restriction enzymes *Eco*RI and *Mse*I for 1 h at 37°C. The digestion mixture (10 µL) contained 0.5–1 µg genomic DNA, 5 U *Eco*RI enzyme, 5 U *Mse*I enzyme and 1 × *Mse*I buffer (Buffer 2, New England Biolabs) and an appropriate amount of water. The complete digestion mixture was ligated with relevant AFLP adapters overnight at room temperature. Each ligation reaction (20 µL) contained the restriction digestion product (10 µL), 1 × T4 DNA ligase buffer with EDTA, 0.05 M NaCl, 0.05 mg mL<sup>-1</sup> BSA, 6 U T4 DNA ligase, 10-pmol *Eco*RI adapter, 100-pmol *Mse*I adapter and appropriate amount of water. The structure of the *Eco*RI-adapter was:

5'-CTCGTAGACTGCGTACC  
CATCTGACGCATGGTTAA-5'

The structure of the *Mse*I-adapter was:

5'-GACGATGAGTCCTGAG  
TACTCAGGACTCAT-5'

Ligation product was diluted 20-fold and used as a template for preselective amplification.

Preselective amplification was performed using specific primers complementary to each adaptor sequence. Each preselective PCR (20 µL) contained 10 µL of 2 × TaqPro Complete mixture with 1.5 mM MgCl<sub>2</sub>, 100 pmol of each primer, 4 µL of 20-fold diluted ligation product and an appropriate amount of water. The cycling profile for preselective amplification was: one cycle of 72°C for 5 min to inactivate restriction and ligation, 30 cycles of 94°C for 40 s, 54°C for 40 s and 72°C for 2.5 min and one final cycle of 72°C for 10 min. Products from preselective PCR were diluted 20-fold and used as templates for selective amplifications. Pairs of selective primers complementary to each adaptor sequence (except for the last three selective nucleotides added at their 3' end) were used for selective PCR.

Each selective PCR (20 µL) contained 10 µL of 2 × TaqPro Complete mixture with 1.5 mM MgCl<sub>2</sub>, 100 pmol of each primer, 5 µL of 20-fold diluted product of preselective amplification and appropriate amount of water. For selective PCR, a touch-down amplification was used: 10 cycles of 94°C for 20 s (denaturation), 66°C for 30 s (annealing), 72°C for 2 min (extension), with a 1°C decrease of annealing temperature each cycle, followed by 20 cycles of amplification at 94°C for 20 s, 56°C for 30 s and 72°C



for 2 min. We analyzed six combinations of primer pairs containing core sequences complementary to EcoRI or MseI adapters with addition of the following selective nucleotide triplets on 3'-end: forward primers EcoRI-ACA and -ACA, and reverse primers MseI-CTC, -CTA, and -CAG. All amplifications were performed in an Eppendorf MasterCycler Gradient thermal cycler (Brinkmann, Westbury, NY).

DNA fragments obtained by selective amplification were resolved on 8% acrylamide gels for 7–8 h in 1 × TBE buffer at 15 °C to improve resolution and were visualized by silver-staining using a method described by Sokolova and Boulding (2004). At least 3 reference samples were run on each gel as internal standards, along with 100 bp and 50 bp DNA ladders (Invitrogen, Carlsbad, CA). AFLP loci were analyzed using the Kodak EDAS 290 gel imaging system and Kodak 1D Image Analysis Software (Kodak, Rochester, NY), and scored as a presence or absence of a fragment of the respective length. Software-generated scores were verified manually. Only fragments between 100 and 350 bp were included into the analysis because shorter or longer fragments could not be reliably scored.

Originally we scored 100 AFLP markers as present or absent in a random sample of oysters, and 48 of these markers proved to be sufficiently polymorphic (neither haplotype with a frequency greater than 80%) for use. These 48 markers (designated *M1* through *M48*) were scored in 35 oysters, although 1 marker was missing in 7 oysters and 4 markers were missing in 5 oysters (all 35 markers were present in 23 of the 35 oysters). This sample of 35 contained only 3 oysters that previously had been classified as not infected with *V. vulnificus* (Sokolova et al. 2005), making the presence/absence of infection an unsuitable qualitative trait for analysis. Instead, we analyzed a quantitative trait, the magnitude of infection of *V. vulnificus* (log of CFU/g counts) among the 32 infected oysters. Because this trait was previously found to be correlated with weight of the oysters (Sokolova et al. 2005), we first used regression to adjust all these values for differences in overall weight. Our sample of 35 oysters included 7 individuals that previously were classified as not infected with *P. marinus*, making it possible to analyze association of AFLP markers with the presence/absence of infection by this parasite. Both dependent variables, the magnitude of *V. vulnificus* infection and the presence/absence of *P. marinus* infection, were tested for their association with the AFLP markers as described below.

AFLP markers are useful in a genome-wide search for gene or gene groups linked with the differential resistance to parasites or pathogens and do not require prior knowledge about the host DNA sequences; they are anonymous and thus can in principle be amplified from DNA of the host or the parasite. In this study, we used oyster gill tissues where the host DNA content is expected to exceed the parasite DNA content by several orders of magnitude because of the relatively low infection intensity (mean score  $3.6 \pm 0.24$  by Mackin scale corresponding to medium infection,  $n = 18$ , Grewal & Sokolova, unpublished data) and large differences in the genome size of the host and the parasites/pathogens (700 Mb in *C. virginica* versus 28 Mb and 5.3 Mb in *P. marinus* and *V. vulnificus*, respectively) (Gregory 2001, Chen et al. 2003; <http://www.tigr.org/tdb/e2k1/pmg/intro.shtml>). Because amplification is a competitive process, the number of copies amplified from rare templates (e.g., from parasite DNA) will be negligible compared with the abundant templates (i.e., from oysters) when the same number of cycles is used, and products from rare templates are not likely to be visualized on the AFLP gel. In this study, we scored

only well-defined AFLP bands of high intensity to ensure that all AFLP markers belong to the oyster DNA.

#### Single-Locus Associations

As a first step in testing for associations of the marker haplotypes with infection with either *V. vulnificus* or *P. marinus*, it was necessary to assess relatedness among the 48 markers themselves. This assessment was accomplished with the MAPMAKER 3.0b program (Lander et al. 1987, Lincoln et al. 1992), which tested for potential linkage groups among these markers. We reduced the default criterion of 3.0 in this program to 2.5 to be conservative with our limited sample size, and the program identified 9 linkage groups, each with 2–4 markers. However, only 22 markers were included in these groups, with the remaining 26 being unlinked and therefore considered to be independent (effective number of total linkage groups =  $9 + 22 = 31$ ).

We used 1-way analyses of variance (ANOVA; Sokal & Rohlf 1995) to test for associations of each of the 48 markers with the degree of *V. vulnificus* or *P. marinus* infections. In these analyses, the AFLP marker was the single factor (with two levels, presence or absence) and the degree of infection was the dependent variable. Each ANOVA yielded the conventional *F* statistic with its associated probability, with any probabilities less than 0.05 indicating conventional significance.

Because 48 ANOVAs were conducted for the analysis of *V. vulnificus* resistance, and another 48 ANOVAs for the analysis of *P. marinus* resistance, it was necessary to adjust the conventional significance level to ensure that the experiment-wise error rate did not exceed 5% (Sokal & Rohlf, 1995). To accomplish this, we used a permutation test (Churchill & Doerge 1994) involving 1,000 iterations in which the infection values (scores for *P. marinus* or *V. vulnificus*) were randomly permuted, merged with the AFLP marker data, and ANOVAs performed. These ANOVAs generated *F* values with their associated probabilities that were logarithmically transformed to produce LOD scores (Lander & Botstein 1989) as follows:  $\text{LOD} = \log_{10}(1/\text{Prob})$ . For each of the 31 linkage groups, the highest LOD scores generated in each permutation run were then ranked, and the 50th and 10th highest values from each distribution represented the 5% and 1% group-wise threshold LOD scores. An experiment-wise threshold value across all linkage groups also was obtained from the 50th (5%) highest LOD scores that were observed on any linkage group during each of 1000 iterations (Churchill & Doerge 1994). If the highest LOD score calculated for a given linkage group exceeded its appropriate 5% group-wise threshold value (or especially the experiment-wise value), the test of association was considered to be significant and suggested that there is a genetic locus influencing resistance to *V. vulnificus* (or *P. marinus*) infection on the chromosomal fragment adjacent to the respective AFLP marker.

#### Two-Locus Associations

We used 2-way ANOVAs to test for significance of pairs of AFLP markers for their potential interactive effects on *V. vulnificus* and *P. marinus* resistance and/or susceptibility. Marker pairs were tested only for the 465 pairwise combinations of the 31 linkage groups because markers within linkage groups are associated. The significance of marker epistasis was indicated by the probability associated with the *F* value for the interaction of each pair of markers in the ANOVAs.

The multiple comparisons problem inherent in this many tests

for epistasis was addressed by first calculating the effective number of independent tests for each linkage group (Cheverud 2000, Cheverud 2001). This was calculated as  $M_e = M(1 - [V_\lambda(M-1)/M^2])$ , where  $M$  is the number of markers scored (nonlinked markers were given an  $M$  value of 1), and  $V_\lambda$  is the variance of the eigen values of the correlation matrix of markers. The total number of independent epistasis tests then was estimated to be the sum of the crossproducts of the effective number of markers for all 465 pairs of linkage groups. This calculation yielded a sum of 814, suggesting that we might expect about  $5\% \times 814 = 41$  tests to be significant at the 5% level (8 at the 1% level and 1 at the 0.1% level) because of chance alone. Thus epistasis was indicated if the number of  $F$  tests of the interactions reaching the conventional 5% level of significance significantly exceeded 41. This procedure also allowed us to test for individual instances of epistasis by correcting our threshold level of significance via the Bonferroni procedure. Specifically, any specific two-gene interaction was considered to be significant at the 10% experiment-wise level when a given probability from the  $F$  test for the interaction of markers reached the 0.1 Bonferroni threshold level of significance of  $0.1/814 = 0.000123$  (Peripato et al. 2002, Leamy et al. 2005).

## RESULTS

### *V. vulnificus* Infection

Three (*M19*, *M28* and *M31*) of the 48 AFLP markers exhibited associations with the degree of *V. vulnificus* infection that reached significance at the conventional 5% level in the ANOVAs. Further, LOD scores associated with the probabilities for two of these markers (*M28* and *M31*) exceeded the 1% group-wise significance threshold level, although neither LOD score exceeded the 5% experiment-wise threshold value of 2.85 (Table 1). The remaining marker, *M19*, narrowly missed significance at the 5% group-wise level. Table 1 also provides means and standard errors of *V. vulnificus* infection associated with the presence/absence of each of the markers. As may be seen, alleles presumably linked to each of these markers act to increase (*M31*) or decrease (*M28*) the degree of infection.

The results of the 2-way marker analyses done to test for epistatic effects on *V. vulnificus* infection produced 64  $F$  values with associated probabilities less than 5%, this being significantly greater than the number (41) expected at this level by chance alone ( $\chi^2 = 12.99$ ,  $df = 1$ ,  $P < 0.001$ ). The number of  $F$  values reaching significance at the 1% level was 12, but this was not significantly greater than 8 expected at this level ( $\chi^2 = 1.55$ ,  $df = 1$ ,  $P > 0.05$ ). One marker combination, *M42* with *M44*, reached significance at the 10% experiment-wise level ( $P = 0.000114$ ), suggesting that the interaction of these two markers significantly affects *V. vulnificus*

infection levels. In general, therefore, results of these analyses indicate that epistatic interactions of unknown genes linked to these AFLP markers have an effect on the infection level of *V. vulnificus* in our sample of oysters.

To assess the relative effect of individual markers and their interaction on the total variability of *V. vulnificus* infection levels, we ran two multiple regressions. A multiple regression of infection scores on the two markers (*M28* and *M31*) reaching group-wise significance generated a multiple coefficient of determination of 27.8% (or when adjusted for the number of parameters, 22.6%). This suggests that variation in these two markers alone accounts for nearly 30% of the variation in *V. vulnificus* infection levels. Addition of the *M42*-*M44* significant interaction increased the amount explained to 41.8% (adjusted value = 35.4%), a significant improvement in fit ( $\chi^2 = 5.77$ ,  $P < 0.05$ ) from the single-locus model containing the 3 markers.

### *P. marinus* Infection

Three markers (*M5*, *M45* and *M47*) exhibited significance at the conventional 5% level in the tests of associations with the presence/absence of *P. marinus* infection. Two of these markers (*M5* and *M47*) reached group-wise significance, but the remaining marker, *M45*, did not. Table 2 shows these markers, their probabilities and LOD scores, the group-wise threshold LOD scores and the percentage of *P. marinus* infection for oysters with the marker present/absent. *M5* has the lowest probability (highest LOD score), and oysters with this marker show nearly 100% infection with *P. marinus* whereas only about one-half of those oysters without the marker are infected. As was the case for *V. vulnificus*, however, none of these markers affecting *P. marinus* reach the experiment-wise level of significance (LOD = 2.85) in the association tests. In a multiple regression, these two markers accounted for 40.0% (36.2% adjusted) of the total variation in the incidence of *P. marinus* infection.

Results of the tests for marker interactions potentially affecting the incidence of *P. marinus* infection produced 57  $F$  values with associated probabilities less than 5%, this being significantly greater than the number (41) expected at this level by chance alone ( $\chi^2 = 6.17$ ,  $df = 1$ ,  $P < 0.05$ ). The number of  $F$  values reaching significance at the 1% level was 14, and this narrowly missed being significantly greater than the 8 expected at this level ( $\chi^2 = 3.81$ ,  $df = 1$ ,  $P > 0.05$ ). No marker combinations reached significance at the 10% experiment-wise level. Thus there is some suggestion of potential interactions of unknown genes affecting the incidence of *P. marinus* infection, but the evidence for this is less than was seen in the *V. vulnificus* analysis.

TABLE 1.

AFLP markers significantly associated with the degree of infection of *V. vulnificus* in the sample of oysters. Listed are the markers and their associated LOD scores, 5% and 1% group-wise threshold LOD scores, and sample sizes, means, and standard errors for *V. vulnificus* infection for the two haplotypes at each marker.

Marker	<i>P</i>	LOD	Threshold LOD		N	Marker present		Marker absent		
			5%	1%		Mean	St. Error	N	Mean	St. Error
<i>M28</i>	0.0114	1.94**	1.23	1.74	12	3.79	0.218	20	4.48	0.139
<i>M31</i>	0.0032	2.49**	1.62	2.24	24	4.37	0.125	7	3.62	0.355

\*\* =  $P < 0.01$

TABLE 2.

AFLP markers significantly associated with the degree of infection of *P. marinus* in the sample of oysters. Listed are the markers and their associated LOD scores, 5% and 1% group-wise threshold LOD scores, and infection percentages for oysters for which the marker is present/absent.

Marker	P	LOD	Threshold LOD		Marker	
			5%	1%	Present	Absent
M5	0.0021	2.68**	1.21	1.86	95.5%	53.9%
M47	0.0339	1.47*	1.36	1.81	55.5%	88.5%

\* =  $P < 0.05$ ; \*\* =  $P < 0.01$

### DISCUSSION

This study demonstrates the feasibility of the AFLP approach to search for genetic markers associated with oyster genes potentially influencing resistance to *V. vulnificus* and *P. marinus*. In fact, the results of association tests using the AFLP markers suggest that there are genes that act directly or indirectly to control the levels of infection by these parasites in oysters. We found two markers that were significantly associated with infection levels of *V. vulnificus* and two others associated with the incidence of infection of *P. marinus*. Further, there was evidence of significant genetic interactions (epistasis) affecting the levels of both pathogens, especially of *V. vulnificus*. The impact of these unknown genes on variability in infection levels was impressive, accounting for about 40% of total variation in each case.

Whereas the above-described results certainly are encouraging, they should be interpreted with caution because of the relatively small sample size used in this pilot study. This is especially so because all marker associations that reached significance were at the group-wise level, and thus may be considered only as suggestive of the presence of the linked functional genes (Lander & Kruglyak 1995) responsible for the control of infection levels. To provide significant evidence for such genes, the LOD scores should have exceeded the more conservative experiment-wise threshold level (Lander & Kruglyak 1995). Therefore, our results would need to be verified with subsequent studies using larger sample sizes as well as oysters from different populations (Kramer et al. 1998). Such verification is clearly required before AFLP markers could be recommended for use in marker-supported breeding programs aimed at selecting for resistant oyster strains. Nonetheless, even with these caveats, our results clearly imply that AFLP analysis is a viable strategy to detect such markers in oyster populations and that a future study using a larger sample size may well detect genes strongly associated with resistance to these two important parasites/pathogens in oysters.

Our results also suggest that beyond single-locus effects of genes, it is important to consider screening for epistatic interac-

tions of genes while searching for loci associated with disease/pathogen resistance in oysters. We found evidence that gene interactions affected infection levels in the oysters, especially of *V. vulnificus* where the M42-M44 marker interaction reached experiment-wise significance in the association tests. This result involves two markers that were different from those (M28, M31) showing individually significant effects and thus would not have been detected had we conducted single-locus analyses only. This sort of result is not at all uncommon, and highlights the role of epistasis in various quantitative traits (Cheverud & Routman 1995, Leamy et al. 2002, Leamy et al. 2005), including disease resistance (Templeton 2000). Therefore, in future studies searching for genetic markers of disease resistance for marker-supported breeding programs, it seems important to take epistatic interactions between the loci into account.

As a corollary, the AFLP approach described in this study provides a cost-effective and relatively rapid method of screening of oyster stock for marker loci (or their epistatically interacting combinations) associated with resistance to two important pathogens affecting the oyster industry—*V. vulnificus* and *P. marinus*. A considerable advantage of this method is that identification of resistant oysters to be used for the parent stock can be performed noninvasively using a small tissue biopsy for DNA extraction and analysis and at a reasonable cost. We believe that the same approach may also be used with other parasites (e.g., *Haplosporidium*) or with invasive organisms such as *Polydora* providing a new tool to combat disease in oyster stocks. In host-parasite systems where the parasite genome size is small (such as in a protozoan *P. marinus* or a bacterium *V. vulnificus*) DNA isolated from the host tissue may be directly used in the AFLP analysis, especially if tissue with low to moderate infection intensity is selected ensuring that the parasite DNA content is negligible compared with the host DNA content. For parasites with large genomes or for very heavily infected tissues, it may be advisable to use markers that predominantly amplify from noninfected host individuals but not from the infected ones (such as M47 and M28 in this study), or to establish AFLP controls using DNA from pure parasite cultures to distinguish between the markers that are amplified from DNA of the host and the parasite.

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## SEASONAL CHANGES IN DRY MASS AND ENERGETIC CONTENT OF *MUNIDA SUBRUGOSA* (CRUSTACEA: DECAPODA) IN THE BEAGLE CHANNEL, ARGENTINA

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**ABSTRACT** *Munida subrugosa* is the most abundant benthic species in the Beagle Channel (55 S, 68 W), Tierra del Fuego. Moreover, this species has two simultaneous but different feeding habits: predator and deposit feeder. Because of its high abundance (100 and 100 m<sup>-2</sup>) and trophic position, this species plays a key role in the subantarctic benthic ecosystems. However, little is known about its energetic content and changes in dry mass during its reproductive cycle. Samples of *M. subrugosa* were obtained in 2000 to 2001 by means of an epibenthic trawl. The relative water content (WC) and the energetic content (EC) (kJ·g<sup>-1</sup> ash free dry mass [AFDM]) of whole adult animals, and the relative dry mass (RDM) and EC of tissues and organs of females were measured throughout one year. The EC investment of adults at the time of maximum gonadal development was evaluated. The EC was measured using a bomb calorimeter. The mean WC and EC for *M. subrugosa* was 59% ± 7% and 19 ± 2 kJ·g<sup>-1</sup> AFDM, respectively. WC and EC for whole adult animals varied significantly throughout the year, attaining maximum values in autumn or summer respectively, after the pattern of seasonal reproduction and feeding. Moreover, the EC of *M. subrugosa* varied by 30% annually. The RDM and EC investment in gonadal development was significant higher in females. The hepatopancreas in *M. subrugosa* is used as an energetic storage organ, because RDM and EC increased before vitellogenesis and moulting.

**KEY WORDS:** subantarctic, Anomura, reproductive cycle, squat lobster, *Munida gregaria*

### INTRODUCTION

The anomuran crab, *Munida subrugosa* (White, 1847) attains length 5–7 cm, and nearly 15-g wet mass, that lives off southern South America, New Zealand and southern Australia (Boschi et al. 1992). In South America, the Beagle Channel represents the southern distributional limit for this species. *Munida subrugosa* constitutes up to 50% of the macrobenthic community biomass and the 85% of the density of anomuran and brachyuran decapods at the eastern entrance to (Arntz et al. 1996, Gorny et al. 1996), and in, the Beagle Channel (Pérez-Barros et al. 2004). The bycatch of the hake fishery from the Atlantic shelf off Argentina during 2000 was dominated by *Munida* spp. and yielded 6674 t (Villarino et al. 2002). A second sympatric and morphological similar species, *M. gregaria* Fabricius 1973 also occurs in the Beagle Channel (Tapella et al. 2002a). The specific identity of both species is still controversial and molecular studies suggest that both are the same species probably undergoing speciation (Pérez-Barros et al. submitted). Hence, results presented in this study may be extensive and valid also for *M. gregaria*.

Currently, the only galatheids that are commercially exploited are *Pleuroncodes monodon* (H. Milne Edwards, 1837) and *Cervimunida johni* Porter, 1903 off Chile, ca. 35 S. Landings peaked 60,000 t in 1972, varied around 10,000 t during the 1980s and between 2,000 and 12,000 t during the 1990s. *Munida* spp. prospectively constitutes an exploitable shellfish at the southern tip of South America (Rayner 1935, Lovrich et al. 1998). High abundance of *Munida* spp. and its potential economical applications are comparable to those of other galatheids. Commercial uses can be as cocktail shrimp, source of natural astaxantins for coloration of cultured salmon or chicken eggs, digestive proteases for cheese manufacture, or milled as source of proteins of balanced food (Auriolles Gamboa & Balart 1995, Lovrich et al. 1998). Meat yield of *M. subrugosa* is 7.5% wet weight (Lovrich et al. 1998).

*Munida subrugosa* plays an important linking role in subantarctic benthic ecosystems, mainly due its trophic position (Romero

et al. 2004). In one respect as predator and deposit feeder, *M. subrugosa* feeds on lower trophic level organisms, crustaceans and algae and on detritus and sediments (Romero et al. 2004). Hence, *M. subrugosa* is responsible for incorporating organic matter into the trophic web by passing saprophytic biodegradation (Romero et al. 2004). On the other hand, *M. subrugosa*, being very abundant, is prey of several top predators. Particularly in the Beagle Channel, galateid crabs are fed on by king crabs, fishes, cormorants, penguins and whales (Romero et al. 2004). *Munida subrugosa* participates in short trophic chains, which are probably efficient in the energetic transfer, because the shorter the chain, the less energetic losses (Pianka 1982). Hence the importance of studies related to energetic contents of this species.

The study of the energetic transfer between populations is an important step in the analysis and understanding of the functioning of an ecosystem. All energy that passes through a population is either transformed to heat or to other forms of energy, or is available to pass to another trophic level. Accurate values for the energetic content of both tissues of organisms or whole organisms are essential for a better understanding of energetic relations in ecosystems. Moreover, measures of whole body energy are useful in quantifying consumption of species by their predators (Paul 1997). Hitherto, the only data for the energetic content of related species, *M. valida* Smith, 1883 and *M. iris* A. Milne Edwards, 1880, are from the deep-sea continental slope (Steimle & Terranova 1988), in the northwest Atlantic.

In decapods, bioenergetic studies of variation in energetic content during the reproductive cycle are scarce. In the shrimp *Crangon crangon* (Fabricius, 1795) a cycle of progressive and retrogressive changes between the dry mass of the ovary and hepatopancreas occurs during ovarian development (Haefner & Spaargaren 1993). Other studies report on both seasonal variations and general descriptions of the lipid content and its relation with the reproductive cycle (Clarke 1977, Teshima et al. 1989, Styris have & Andersen 2000, Wen et al. 2001, Mourente & Rodríguez 1991).

This study analyzes temporal variation in the dry mass and energetic content of *Munida subrugosa* in the Beagle Channel

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TABLE 1.

Predictive regression of relative dry mass (RDM) on crab size (carapace length, CL, in mm) in different sexes and ovigerous condition of *Munida subrugosa*.

Sex and Condition	Equation of Regression	CL Range (mm)	N	R <sup>2</sup>	F	P
Non-ovigerous females	$\text{Log RDM} = 2.5 \log \text{CL} - 3.09$	13–25	106	0.75	316.5	$\leq 0.001$
Ovigerous females	$\text{Log RDM} = 2.7 \log \text{CL} - 3.24$	15–26	45	0.72	106.8	$\leq 0.001$
Males	$\text{Log RDM} = 3.0 \log \text{CL} - 3.71$	16–24	151	0.78	545.4	$\leq 0.001$
	$H_0: b_{\text{NOF}} = b_{\text{OF}} = b_{\text{M}}$				3.5	0.031
	$H_0: b_{\text{NOF}} = b_{\text{OF}}$				0.2	0.700
	$H_0: b_{\text{NOF}} = b_{\text{M}}$				6.7	0.010
	$H_0: b_{\text{OF}} = b_{\text{M}}$				1.6	0.210

References: NOF and OF, non-ovigerous and ovigerous females, respectively. N, sample size;  $r^2$  coefficient of determination. M, males; F,  $F$ -statistic.

throughout a year. The dry mass and energetic content of females and males is compared on a monthly basis. In females, the energetic changes in the ovary, hepatopancreas and abdominal muscle are analyzed on a bimonthly basis. Furthermore, the energetic investment of females and males at the maximum stage of gonadic development before mating is documented.

## MATERIALS AND METHODS

### Study Site and Sampling

The field work was carried out in Bahía Ushuaia, in the Beagle Channel (55° S; 68° W). Samples of *M. subrugosa* were obtained from waters shallower than 40-m depth with an epibenthic trawl net of 10-mm mesh size and 1.7-m mouth width (see Tapella 2002 for details). Crabs used for the analyses were preserved alive on-board, and dissected or frozen at  $-20^\circ\text{C}$  on arrival at the laboratory.

The standard measure of body size, the carapace length (CL), was determined to the nearest 0.1 mm with a dial caliper. Crabs >10 mm CL are gonadal mature and therefore considered as adult individuals (Tapella et al. 2002b) as such, only crabs of >10 mm were used for this study. Average sizes for females and males used in this study were  $19.9 \pm 2.6$  and  $19.9 \pm 2.4$  mm CL, respectively, which is coincident with the modal sizes of the Beagle Channel population (c.f. Tapella 2002). Water content (%) and energetic content (EC) of whole *M. subrugosa* individuals, both males and females, were studied from monthly samples obtained between February and December 2000. From the total monthly catch, 12 males and 12 females were randomly selected and frozen.

The relative dry mass (RDM) and EC of different organs or tissues of female *M. subrugosa* were studied from samples taken once every second month from December 2000 to November 2001. At each sampling, 8–22 females were randomly selected from the total catch for analysis. Animals were kept in tanks with running seawater for a few hours until the dissection, then the carapace was removed, and ovaries, hepatopancreas and abdominal muscle were dissected.

To compare the energetic investment in reproduction in males and females, an additional sample of 15 male *M. subrugosa* was taken in May of 2001, when gonadosomatic indexes are near to reach their maximal values (Tapella et al. 2002b). Both testicles and vasa deferentia were used for the energetic measurements.

### Water Content and Dry Mass Determination

Wet mass (WM) of the whole animals, stomach contents, hepatopancreas, abdominal muscles and ovaries of females, and testicles and vasa deferentia in males were recorded to 0.0001 g

precision. Samples were dried to constant weight at  $60^\circ\text{C}$ , and the dry mass (DM) recorded.

The percentage water content of the animals was calculated as  $(\text{WM} - \text{DM}) / \text{WM} \times 100$ . Because the DM is dependent on the animal size, we calculated the RDM of organs and tissues, which was the  $\text{DM}_{\text{organ}} / \text{DM}_{\text{whole animal}}$ . The RDM of all animals was calculated from the animal size (CL) and according to their sex and ovigerous condition. Lineal regressions between log RDM and log CL were calculated from a different sample of several nonovigerous females, ovigerous females and males (Table 1), obtained in July and December of 2001. The equality of the slopes was checked with an analysis of covariance (Sokal & Rohlf 1995).

### Calorimetric Determination

After obtaining the DM, samples were ground and pelletized with a press model Parr 2812. The caloric content of each sample was obtained by burning pellets of 20–200 mg in a calorimeter Parr model 1425 with a microbomb Parr model 1341. Energy values were calculated using standard equations (Parr Instrument Co. 1991, 1993). The values obtained were corrected for ash and acid content and were expressed as  $\text{kJ} \cdot \text{g}^{-1}$  ash free dry mass (AFDM). Benzoic acid calibrations were done periodically.

## RESULTS

The ingestion in *Munida subrugosa* was clearly seasonal, being maximum in December (Fig. 1; ANOVA  $F = 25.4$ ,  $P < 0.001$ ).

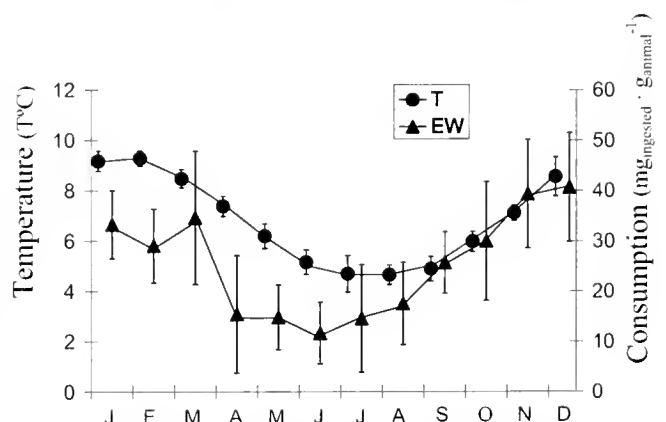


Figure 1. Average monthly temperatures of the Beagle Channel ( $^\circ\text{C}$ ) and average quantity of food (EW,  $\text{mg}_{\text{ingested}} \cdot \text{g}_{\text{animal}}^{-1}$ ) in the stomach contents of adult *Munida subrugosa*. Temperatures are historical monthly averages of the sea surface temperature, 1985 to 1998 (Balestrini et al. 1998).

Food uptake in *M. subrugosa* follows the same pattern as surface water temperature (Fig. 1:  $r = 0.66$ ,  $P = 0.02$ ): in autumn and winter, ingestion was almost 4 times less than in spring or summer.

For the whole adult animals values of water content and EC of male and female *M. subrugosa* varied significantly throughout the year (Fig. 2, Table 2). Overall, the mean water content and EC for both sexes were  $59\% \pm 7\%$  and  $19 \pm 2 \text{ kJ} \cdot \text{g}^{-1}$ , respectively. The minimum and maximum values of water content were registered in autumn and winter, respectively (Fig. 2A). The minimum of EC was recorded in winter (Fig. 2B). The interaction between month and sex for the water content was statistically significant (Table 2). Particularly, the water content of females was the lowest in June (ANOVA  $F = 6.24$ ,  $P = 0.01$ ).

#### RDM and EC in Organs and Tissues

##### Ovaries

The ovarian RDM and EC varied significantly throughout the year (Fig. 3, Table 3). Both RDM and EC reached their maximum values in May; afterwards, RDM and EC gently decreased and in November attained values similar to December (Fig. 3).

##### Hepatopancreas

The hepatopancreas RDM and EC varied significantly throughout the year (Fig. 3, Table 3). RDM and EC showed two maximum values around  $140 \pm 40 \text{ mg}_{\text{org}} \cdot \text{g}_{\text{animal}}^{-1}$  and  $32 \pm 2 \text{ kJ} \cdot \text{g}^{-1}$ , respectively, in March and November (Fig. 3). No significant differences in RDM and EC between these months were found (Tukey test,  $P = 0.33$  and  $P = 0.99$  for RDM and EC, respectively).

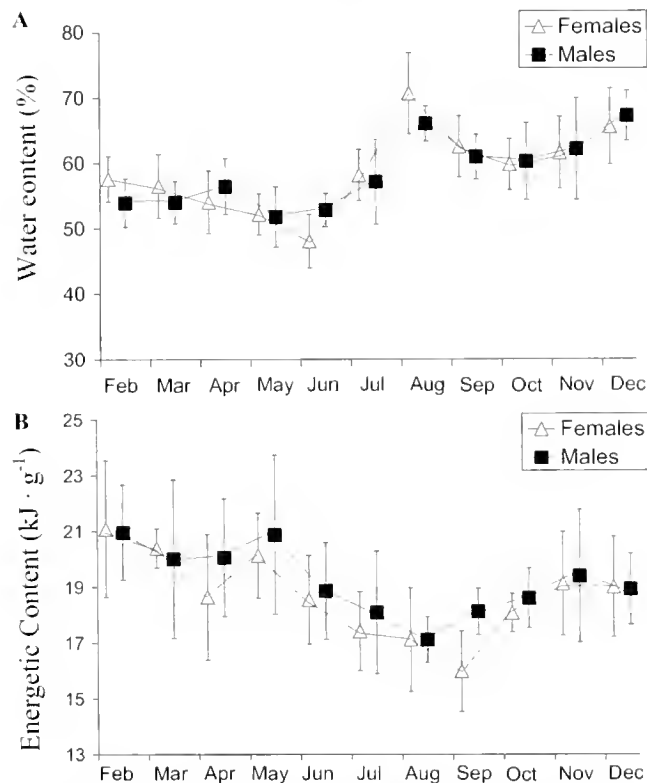


Figure 2. Mean monthly percentage of water content ( $\pm$ SD) (A) and energetic content ( $\text{kJ} \cdot \text{g}^{-1}$  AFDM) ( $\pm$ SD) (B) of adult *Munida subrugosa* from the Beagle Channel between February and December 2000. Monthly sample size was 12 females and 12 males.

TABLE 2.

Two-way analyses of variance (ANOVA) to detect differences in percentage of water content and energetic content ( $\text{kJ} \cdot \text{g}^{-1}$ ) in adult *Munida subrugosa* between sexes and throughout the year.

Source	Water Content (%)			Energetic Content		
	d.f.	MS	F	d.f.	MS	F
Month	10	776.36	35.5*	10	41.63	12.66*
Sex	1	10.43	0.5	1	16.65	5.06*
Month $\times$ sex	10	45.53	2.1*	10	3.30	1.01
Error	242	21.87		242	3.29	

References: MS, mean square; EC, energetic content; F, F-statistic. Significant differences ( $P < 0.05$ ) are indicated by asterisk.

##### Abdominal Muscle

The abdominal muscle RDM varied significantly throughout the year (Fig. 3, Table 3), principally because of a single maximum RDM recorded in March ( $80 \pm 10 \text{ mg}_{\text{org}} \cdot \text{g}_{\text{animal}}^{-1}$ ). This value duplicated the others recorded during the study period. The EC of the abdominal muscle was similar throughout the year (Fig. 3, Table 2), with an annual mean EC of  $22 \pm 2 \text{ kJ} \cdot \text{g}^{-1}$ .

#### Relationship Among Ovary, Hepatopancreas and Abdominal Muscle

Maxima in RDM of hepatopancreas and muscle and in EC of the hepatopancreas preceded that of the ovary (Fig. 3). In terms of

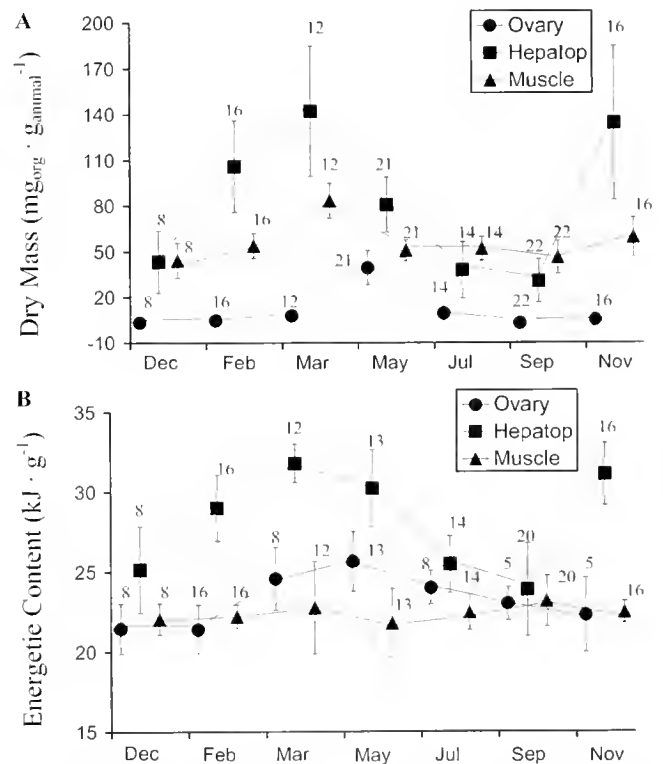


Figure 3. Mean relative dry mass ( $\text{mg}_{\text{org}} \cdot \text{g}_{\text{animal}}^{-1}$ ) ( $\pm$ SD) (A) and energetic content ( $\text{kJ} \cdot \text{g}^{-1}$  AFDM) ( $\pm$ SD) (B) of ovaries, hepatopancreas and abdominal muscles of females *Munida subrugosa* from Beagle Channel between December of 2000 and November of 2001. Note that each month symbols are apart to avoid overlapping (sampling was done on the same date). Numbers on top of each point are samples sizes.



TABLE 3.

One-way analysis of variance (ANOVA) to detect differences in relative dry mass ( $\text{mg}_{\text{org}} \cdot \text{g}_{\text{animal}}^{-1}$ ) (RDM) and energetic content ( $\text{kJ} \cdot \text{g}^{-1}$  AFDM) (EC) of three different tissues of female *Munida subrugosa* throughout the year.

Source	Relative Dry Mass				Energetic Content		
	d.f.	MS	F		d.f.	MS	F
Ovary	6	3,345.51	114.02	*	6	29.52	10.70
Error	102	29.34			55	2.76	
Hepatopancreas	6	32,192.20	36.47	*	6	150.07	29.91
Error	102	882.63			91	5.02	
Abd. muscle	6	2,193.30	22.36	*	6	3.09	1.19
Error	102	98.08			89	2.59	

References: Abd. muscle, abdominal muscle; F, F-statistic. Significant differences ( $P < 0.05$ ) are indicated by asterisk.

RDM, the hepatopancreas and muscle were positive and significantly correlated (partial correlation,  $r_{\text{H M}} = 0.82$ ,  $n = 109$ ,  $P < 0.05$ ). However, in terms of EC the hepatopancreas and muscle were uncorrelated (partial correlation,  $r_{\text{H M}} = 0.088$ ,  $n = 59$ ,  $P > 0.05$ ). Hepatopancreas and ovary RDM were positive and significantly correlated (partial correlation,  $r_{\text{H O}} = 0.55$ ,  $n = 109$ ,  $P < 0.05$ ), whereas the EC of these organs was positive but not significantly correlated (partial correlation,  $r_{\text{H O}} = 0.178$ ,  $n = 109$ ,  $P > 0.05$ ). Ovary and muscle RDM and EC were negatively correlated, and none of these correlations were significant (partial correlation,  $r_{\text{O M}} = -0.31$ ,  $n = 109$ , and  $r_{\text{O M}} = -0.184$ ,  $n = 59$ , both  $P > 0.05$ , respectively).

#### Gonad Energy and Mass at the End of the Gonadal Development

The gonad RDM was significantly higher in females ( $40 \pm 10 \text{ mg}_{\text{org}} \cdot \text{g}_{\text{animal}}^{-1}$ ) than in males ( $6 \pm 2 \text{ mg}_{\text{org}} \cdot \text{g}_{\text{animal}}^{-1}$ ) (Student  $t = 12.91$ ,  $P < 0.001$ ). The gonad EC was also significantly higher in females ( $26 \pm 2 \text{ kJ} \cdot \text{g}^{-1}$ ) than in males ( $22 \pm 2 \text{ kJ} \cdot \text{g}^{-1}$ ) (Student  $t = 4.20$ ,  $P = 0.002$ ). Hepatopancreatic RDM ( $80 \pm 28 \text{ mg}_{\text{org}} \cdot \text{g}_{\text{animal}}^{-1}$ ) and EC ( $30 \pm 3 \text{ kJ} \cdot \text{g}^{-1}$ ) were similar in both sexes (Student  $t = 0.22$ ,  $P = 0.82$ , and  $t = -1.07$ ,  $P = 0.30$ , respectively). Similarly, muscle RDM ( $50 \pm 9 \text{ mg}_{\text{org}} \cdot \text{g}_{\text{animal}}^{-1}$ ) and EC ( $21 \pm 2 \text{ kJ} \cdot \text{g}^{-1}$ ) were also similar in both sexes (Student  $t = -0.08$ ,  $P = 0.93$  and  $t = 0.56$ ,  $P = 0.58$ , respectively).

#### DISCUSSION

Our results depict a typical annual energetic cycle of a decapod in subantarctic latitudes (Thatje 2004 and references therein). Over the year, variations in energetic content can be divided into three different periods. During summer and autumn, the total energetic content is fairly constant (Fig. 2B), yet with a high flow of matter and energy from the hepatopancreas and muscle towards ovaries (Fig. 3B). Growth of the ovary is indicated by a maximum RDM and EC in May (Fig. 3A), which coincides with the lowest water content in the whole individuals (Fig. 2A). Subsequently during winter, the total energy content decreases after the egg-extrusion (c.f. Tapella et al. 2002b), and is coincident with a period of low ingestion (Fig. 1) and the lowest values of energy and mass in organs and tissues (Fig. 3). Finally, the energy accumulation phase occurs during the spring (Fig. 3B), when the food intake increases (Fig. 1), and the hepatopancreas grows for a second time in the

year (Fig. 3). We attribute these variations to a seasonal feeding pattern and ultimately to physiological events such as reproduction.

The seasonal variations in somatic energetic content of *M. subrugosa* probably reflect its temporal feeding patterns. Development and physiological events in ectotherms from high latitudes may be regulated by the seasonal availability of food, and the degree of food dependence given their position in the food web (Clarke 1988). Particularly, the environment of the Beagle Channel is subantarctic with a marked seasonality in surface temperature ( $4.5^\circ\text{C}$ – $9^\circ\text{C}$  in August and January, respectively) and photoperiod (18:6 light:dark in summer and *vice versa*). *Munida subrugosa* feeds on small macroalgae, small crustaceans and particulate organic matter (Romero et al. 2004). Food uptake in *M. subrugosa* follows the same pattern as water temperature. In turn, this could also reflect the different seasonal availability of producers (macroalgae and phytoplankton) and other prey with an annual life span.

In female *Munida subrugosa* maximum somatic energetic values recorded between February and May can be related to the reproductive cycle. In February, oocytes begin their secondary vitellogenesis (i.e., yolk accumulation) (Tapella et al. 2002b). This coincides first, with an increase in EC and RDM of the hepatopancreas in February and March and second with the increase in ovary EC during March and May. The ovary RDM drastically drops in July, right after the egg extrusion in June (Tapella et al. 2002b). Furthermore, the ovary EC gently decreases after egg extrusion, probably because of the presence of nonextruded oocytes and the reorganization of nutritional material associated with vitellogenesis (c.f. Johnson 1980).

The water content of *M. subrugosa* presents two maxima, in August and December. The latter is coincident with one of the peaks in RDM and EC of the hepatopancreas. These changes can be attributed to the moulting period. Tapella (2002) showed that *M. subrugosa* >10 mm CL has 2 periods of moult during the year, in spring and in summer. At moulting, decapods absorb a great quantity of water, which is later replaced by the organic matter that constitutes tissues (Vernet & Charmantier-Daures 1994). Nevertheless, the hepatopancreatic energetic increase during February is probably associated with the energy gained for summer moulting and ovarian growth.

*Munida subrugosa* uses its hepatopancreas to store energy, because this organ increases its RDM and EC before vitellogenesis (peaking in March) and moulting (peaking in November). Flexibility in storage and mobilization of nutrient reserves confers an evolutionary advantage, enabling an organism to stay active during periods of starvation (Crawford 1979), such as the moulting period (O'Halloran & O'Dor 1988). In crustaceans, the hepatopancreas is the principal energy supplier, and a large part of this energy is stored as lipids (e.g., Chapelle 1977, Mourente & Rodríguez 1991, Albessard et al. 2001, Ravid et al. 1999). The main functions of these stored lipids are as reserves for vitellogenesis and moulting (Dall 1981). The material accumulated by the hepatopancreas is converted to other compounds that are released into the hemolymph for an eventual use by the ovary (Haefner & Spaargaren 1993). In high latitude species, the energy stored in the hepatopancreas may be also used during winter when less food is available (Styrishave & Andersen 2000); this holds true for *M. subrugosa*, where reduced food consumption during winter (Fig. 1) is accompanied by the utilization of energy stored in the



hepatopancreas (Fig. 3B) and a general decline in its energetic content (Fig. 2B).

Somatic energetic values of *Munida subrugosa* in the Beagle Channel compare with those of other related species and benthic crustaceans in general. During summer, spring and autumn, somatic EC of *M. subrugosa* was 20.6, 18.9 and 19.5 kJ·g<sup>-1</sup>, respectively. These values are similar to other galatheids from the continental slope of the northwest Atlantic. *Munida valida* presents values of 19.9 kJ·g<sup>-1</sup> in summer; *M. iris* has 19.0 kJ·g<sup>-1</sup> in spring, and *Etmmunida picta* Smith 1883, has 20.6 kJ·g<sup>-1</sup> in autumn (Steimle & Terranova 1988). The mean EC for *M. subrugosa* from the Beagle Channel was 18.9 ± 1.4 kJ·g<sup>-1</sup>, similar to the decapod value from the English Channel of 20.5 ± 1.5 kJ·g<sup>-1</sup> (Dauvin & Joncourt 1989) but significantly lower than the reported EC of 22.7 kJ·g<sup>-1</sup> for benthic crustaceans and the general somatic EC of 23 kJ·g<sup>-1</sup> for macrobenthic invertebrates (Brey et al. 1988).

The somatic EC of *M. subrugosa* varies throughout the year between 16 and 21 kJ·g<sup>-1</sup>. Hence, calculations of consumption rates of predators should consider these seasonal variations carefully. Similarly, studies on energetic budgets of prey of *M. subrugosa* should take into account such seasonal variations. *Munida subrugosa* is one of the main prey of about 30 species of top predators in the Southwestern Atlantic (Romero et al. 2004). In the Straits of Magellan and its channel system, the density of *M. subrugosa* is as high as 27 individuals·m<sup>-2</sup> (Gutt et al. 1999). The Beagle Channel presents the highest biomass and productivity of macrozoobenthos of the Magellanic Region (Thatje & Mutschke 1999). Particularly at <40 m depth, the biomass of *M. subrugosa* is on average 3.4 g·m<sup>-2</sup> (Tapella et al. 2002a), which implies 64 kJ·m<sup>-2</sup> of energy easily accessible to predators above

the sea-bottom. This characteristic is important from an ecological point of view, because *Munida* spp. are part of numerous trophic chains; for example, in the Beagle Channel food consumption among populations of nine seabird species is high, and estimated to be between 1.71 and 3.42 t·d<sup>-1</sup> (Raya Rey & Schiavini 2001). Hence, *M. subrugosa* probably contribute a large proportion of the high energetic flow towards the seabirds, especially diving species like penguins and cormorants. A contrasting potential prey species in the same location is the clam *Eurhomalea exalbida* (Chemnitz, 1795), which lives buried 20 cm in the substrate and is very abundant. Patches of this clam have an average abundance of 83 ind·m<sup>-2</sup> and an annual biomass of 186.4 g·m<sup>-2</sup> (Lomovasky et al. 2002), which represents 3914.4 kJ·m<sup>-2</sup>, 60-fold that of *M. subrugosa*. *Eurhomalea exalbida* is a filter feeder that consumes particulate organic matter. This food is converted into clam tissue and retained under the sea bottom and is unavailable to predators. This clam is a long-lived species, with a life-span of 70 y (Lomovasky et al. 2002). Animals with such life-strategies capture and retain nutrients in living tissue for decades, so that energy transfer to other trophic levels is very low compared with *Munida* spp.

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## THE INTERACTION OF SALINITY AND NA/K RATIO IN SEAWATER ON GROWTH, NUTRIENT RETENTION AND FOOD CONVERSION OF JUVENILE *LITOPENAEUS VANNAMEI*

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**ABSTRACT** The effects of salinity, Na/K ratio and their interaction on growth, molting, nutrient retention and food conversion of *Litopenaeus vannamei* were investigated. The test shrimp were reared in artificial seawater and fed fresh polychaete worms, *Neanthes japonica* (Izuka) for 30 days. Two salinities (30 and 15 ppt) and 5 Na/K ratios (25.6, 34.1, 47.3, 102.1 and 153.3, mmol/mmol) were set, and each treatment had four replicates. During the feeding trial, the molting frequency (MF) was affected by neither salinity nor interaction effects, but Na/K ratio had an effect ( $P < 0.05$ ). And there was a tendency that MF rose with the increment of Na/K ratio at both salinities. Final body weight, weight gain (WG) and specific growth rate (SGR) were significantly influenced by salinity, Na/K ratio and interaction effects ( $P < 0.05$ ), whose mean values at salinity 15 ppt were significantly higher than those at 30 ppt. At 30 ppt, WGs and SGRs of shrimp exposed to Na/K ratio 34.1 and 47.3 were respectively more than 26% and 15% higher than of those exposed to the other ratios ( $P < 0.05$ ), but no significant differences in growth were found among treatments at 15 ppt ( $P > 0.05$ ). Na/K ratio and interaction effects significantly affected the nutrient retention (PR and ER), food conversion (FCE) and protein utility (PER) of the shrimp ( $P < 0.05$ ), whereas salinity showed little impact. At 30 ppt, the PR and ER of shrimp exposed to Na/K ratio 153.3 were more than 30% lower than of those exposed to the other 4 ratios ( $P < 0.05$ ); the FCEs of shrimp exposed to Na/K ratio 34.1 and 47.3 were more than 20% higher than of those exposed to Na/K ratio 25.6 and 102.1 ( $P < 0.05$ ), and the FCE of shrimp exposed to Na/K ratio 153.3 was even lower ( $P < 0.05$ ). At 15 ppt, no significant differences in FCE among treatments were found ( $P > 0.05$ ). PER showed the similar tendency as FCE at both salinities. It was indicated that the shrimp were more adaptable to abnormal Na/K ratios at low salinity than at higher salinities, and good growth could always be obtained within a Na/K range of 34.1–47.3 (mmol/mmol) regardless of salinity.

**KEY WORDS:** salinity, Na/K ratio, interaction, *Litopenaeus vannamei*, growth

### INTRODUCTION

Inland production of shrimp using water from saline aquifers is providing an alternative to traditional coastal aquaculture and a diversification of agriculture, and it is currently undertaken in the United States, Ecuador, Brazil, China and several other countries (Boyd 2002, McGraw et al. 2002). Until the year 2003, inland shrimp farming was present in 25 of the 31 Chinese provinces and autonomous regions, and the annual production of *L. vannamei* cultured in inland has amounted to 296,300 tons in China (Liu et al. 2004).

Although the shrimp farming technology in seawater has reached a high level, it could not be introduced to inland saline water culture directly. Comparative to seawater, the ion profile has changed a lot, and the rule of constancy of composition of seawater does not apply to inland saline water. Furthermore, the ionic composition and salinity of ground water among places can vary markedly; the natural saline water resources in many inland places could not be used in shrimp culture directly (Boyd 2002, Davis et al. 2002, Saoud et al. 2003). For example, In the saline-alkaline area of Yellow River Delta in China, the saline ground waters are chloride type, salinity varies between 5–15 ppt and most of their chemical compositions are similar to that of oceanic seawater of the same salinity except potassium, which is 90% to 95% less than similar salinity oceanic seawater, and shrimp could not survive (Wang et al. 2001, Li et al. 2002).

Current data suggests that salinity and proper ionic composition of saline water are the two necessities for culture suitability evaluations. Numerous reports focus on effects of salinity on shrimp culture (Dalla Via 1986a, 1986b, Huang 1983, Bartlett et al. 1990,

Bray et al. 1994, Vinod et al. 1996, Chen et al. 1996, Ponce-Palafox et al. 1997, Rosas et al. 2001). More studies have begun concerning the ionic imbalance of saline water, especially on supplementing potassium into potassium deficient saline water for shrimp and marine fish culture (Liu 2001, Fielder et al. 2001, Allan & Fielder 2002, McGraw & Scarpa 2003). In addition, Forsberg et al. (1996) reported that the survival of red drum *Sciaenops ocellatus* was significantly corrected with the Na/K and K/Cl ratios of the saline ground water. Zhu et al. (2004) did special experimental work on the effects of Na/K ratio in seawater on growth and energy budget of juvenile *L. vannamei*, and they found that Na/K ratio had significant effects on the survival, molting, growth and energy budget of the shrimp at salinity 30 ppt. However, salinity fluctuation and ionic imbalance often occur simultaneously in saline ground water, and they might interact on the aquatic animals. The aim of this research is to evaluate the interaction of salinity and Na/K ratio in seawater on the growth of *L. vannamei* under laboratory conditions, thus to further the knowledge on shrimp farming with inland saline ground water.

### MATERIALS AND METHODS

#### *Source and Acclimation of Juvenile Litopenaeus vannamei*

The experiment was carried out between June 14 and July 13, 2003. Juvenile *L. vannamei* were obtained from the Jiaozhou Shrimp Farm in Qingdao, China. When the shrimp were transported to the laboratory, one half was stored in two continuously aerated 600-l fiberglass tanks with natural seawater (29–31 ppt), and the other half was stored with diluted seawater (15 ppt) to undergo a 10-day acclimation to the indoor laboratory conditions, during which they were fed *ad libitum* twice a day (8:00 and

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18:00) with commercial shrimp ration pellets (composition: 41.58% crude protein, 8.36% crude lipid and 8.74% moisture; energy content: 19.37 kJ/g dry matter).

#### Experimental Design and Artificial Seawater Preparation

To eliminate the interference of imbalance from other ions, the experimental water was prepared by adding instant artificial sea-salts into fully aerated tap water. The instant artificial sea-salts were specially designed and produced by General Sea Salt Factory, Ocean University of China, in which the sodium and potassium ingredients were precisely compounded so as to keep their total concentration constant, whereas the Na/K ratios varied. The salinity of the newly prepared artificial seawaters was 30 ppt and 15 ppt and pH 8.2. At each salinity level, the total concentration of mono-valent cations and other ions were kept approximately constant. Based on the former experiment result (Zhu et al. 2004), 5 Na/K ratios were set: 25.6, 34.1, 47.3, 102.1 and 153.3 (mmol/mmol), in which 47.3 was identical with the Na/K ratio of oceanic seawater, and it was set as control. The concentrations of Na<sup>+</sup> and K<sup>+</sup> were determined with an inductively coupled plasma-atomic emission spectrophotometer (ICP-OES: VISTA-MPX, VARIAN). The details are given in Table 1.

After the 10-day acclimation and 24 h starvation, shrimp of similar size were selected and weighted individually. To remove excess moisture, shrimp were blotted dry with paper towel and weighted to the nearest 0.001 g using an electronic balance. Two-hundred shrimp that weighted  $1.554 \pm 0.004$  g (mean  $\pm$  SE) were selected, and 160 were randomly assigned to 40 glass aquaria (45  $\times$  25  $\times$  30 cm, 4 individuals/aquarium) to take the 30-day feeding trial. The aquaria were filled with 30 L artificial seawater of different salinities (15 ppt and 30 ppt) and Na/K ratios (25.6, 34.1, 47.3, 102.1 and 153.3). Therefore, 10 treatments, 4 replicates per treatment were conducted. The aquaria were randomly located. To prevent the shrimp from jumping out, every aquarium was covered with a mesh cover. The ambient temperature was controlled with an air-conditioner. Aeration was provided continuously and two-thirds of the water volume was exchanged every second day to ensure high water quality. During the experiment, dissolved oxygen was maintained above 6.0 mg/L, pH  $8.1 \pm 0.2$ , water temperature at  $25^\circ\text{C} \pm 0.5^\circ\text{C}$ , and a simulated natural photoperiod (14 light: 10 dark) was used. The remaining 40 shrimp were dried in an oven at  $65^\circ\text{C}$  to constant weight, homogenized and stored at  $-20^\circ\text{C}$  to estimate the body composition and energy content of the initial shrimp. During the feeding trial, the shrimp were fed fresh polychaete worms, *Neanthes japonica* (Izuka), which had been considered to be the best natural diet in prompting shrimp growth and was widely used in shrimp farming in China (Bi et al. 1995, Zhou & Xie 1995). The shrimp were fed at satiation level twice a day (6:00 and 16:00).

#### Samples Collection and Analysis

During the course of the experiment, the daily food (polychaete worms) supplied was blotted dry with paper towel and precisely weighed and recorded. The uneaten food and feces were collected by siphon within 3 h after each meal. Exuviae (molted exoskeletons) were collected and recorded at all times. The collected uneaten food, feces and exuviae were dried at  $65^\circ\text{C}$  and kept for further analysis. At the end of the experiment, all the test shrimp were starved for 24 h and then collected and dried at  $65^\circ\text{C}$  to constant weight. The shrimp from the same aquarium were pooled as a sample.

Nitrogen content was measured using Kjeltac Auto System 2200 (Foss, Sweden), and crude protein content was calculated from nitrogen content by multiplying 6.25 (AOAC 1984). Crude lipid was determined by the Soxhlet method (AOAC 1984). The energy content of dried samples was determined by Parr 1281 Oxygen Bomb Calorimeter (PARR Instrument Company, USA). Analyses of each sample were conducted in triplicates. The body composition of polychaete worm was analyzed in the same way as the shrimp samples and found to be: 83.6% moisture, 77.8% (dry matter) crude protein and 7.2% (dry matter) crude lipid; energy content was 21.31 kJ/g dry matter.

#### Calculation and Data Analysis

Weight gain (WG), specific growth rate (SGR), molting frequency (MF), feeding rate (FR), food conversion efficiency (FCE) and protein efficiency ratio (PER) were calculated as follows:

$$\text{WG (\%)} = 100(W_2 - W_1)/W_1$$

$$\text{SGR (\%}\cdot\text{day}^{-1}) = 100(\ln W_2 - \ln W_1)/T$$

$$\text{MF (\%}\cdot\text{day}^{-1}) = 100N_m / (N_s \times T)$$

$$\text{FR (\% body weight}\cdot\text{day}^{-1}) = 100C / [T (W_2 + W_1)/2]$$

$$\text{FCE (\%)} = 100(W_2 - W_1)/C$$

$$\text{PER} = (W_2 - W_1)/(C \times \text{Protein content})$$

Apparent energy or protein retention levels (ER and PR, respectively) were calculated as:  $[(W_2 \times (\text{final energy or protein content}/100)) - (W_1 \times (\text{initial energy or protein content}/100))]/C \times (\% \text{ energy or protein in food}/100)$  (Hardy 1989).

Where  $W_2$  and  $W_1$  are the final and initial wet body weight of the shrimp,  $N_m$  is the number of molts,  $N_s$  is the number of shrimp,  $T$  is the duration of the experiment, and  $C$  is the total food consumed.

Statistics were performed with SPSS 10.0 statistical software (SPSS Inc., 1999). The assumption of homogeneity of variances was tested for all data, which were  $\sin^{-1}$ -transformed if necessary. Two-way analysis of variance (ANOVA) was used to test for the

TABLE 1.  
Concentrations of Na<sup>+</sup>, K<sup>+</sup> (mmolL<sup>-1</sup>) and Na/K ratios of the experimental artificial seawater.

Na/K ratio Salinity (ppt)	25.6		34.1		47.3		102.0		153.3	
	30	15	30	15	30	15	30	15	30	15
Na <sup>+</sup>	393.2	197.6	398.0	199.5	402.0	201.0	408.0	203.3	409.5	204.0
K <sup>+</sup>	15.4	7.7	11.7	5.9	8.5	4.2	4.0	2.0	2.7	1.3
Na <sup>+</sup> + K <sup>+</sup>	408.6	205.3	409.7	205.4	410.5	205.2	412.0	205.3	412.1	205.3

interaction of salinity and Na/K ratio in seawater on all data. Significant ANOVAs were followed by a Student-Neumann-Keuls multiple comparison test to locate differences between groups. Significance was accepted when  $P < 0.05$ .

## RESULTS

### Survival and Molting

The survival and molting data of the test shrimp are presented in Table 2. During the 30-day experiment, there were 2 shrimp that died within one aquarium at salinity 30 ppt and Na/K ratio 25.6, and another 2 shrimp died while exposed to Na/K ratio 25.6 and 102.1 at salinity 15 ppt. However, no significant differences in survival among treatments were found ( $P > 0.05$ ).

No significant interaction effects in molting frequencies (MF) were found, whereas the Na/K ratio had an effect ( $P < 0.05$ ). At salinity 30 ppt, MFs for shrimp exposed to Na/K ratio 102.1 and 153.3 are much higher than for those exposed to lower Na/K ratios, even significantly higher than for the control (Na/K = 47.3) ( $P < 0.05$ ). No significant differences in MFs were found among treatments at salinity 15 ppt, but it could be seen that MF increases, whereas Na/K ratio becomes higher.

### Growth

At the beginning of the feeding trial, the body weights of the test shrimp under each treatment were similar (Table 2). At the end of the experiment, the final body weight of the shrimp were significantly influenced by salinity, Na/K ratio and interaction effects ( $P < 0.05$ ). At salinity 30 ppt, the mean final body weight for shrimp at Na/K ratio 153.3 was significantly lower than for those at the other four Na/K ratios ( $P < 0.05$ ), yet no significant differences in final body weight were found between shrimp exposed to the latter four ratios ( $P > 0.05$ ). Shrimp at salinity 15 ppt had significantly higher mean final body weight than at salinity 30 ppt

( $P < 0.05$ ). Na/K ratio showed no effects on shrimp final body weight at 15 ppt ( $P > 0.05$ ), although it did at 30 ppt ( $P < 0.05$ ). The mean final body weight for shrimp exposed to Na/K ratio 34.1 and 47.3 was higher than for those exposed to the other three ratios at both salinities.

Weight gain (WG) and specific growth rate (SGR) both were significantly affected by salinity, Na/K ratio and interaction effects ( $P < 0.05$ ). At salinity 30 ppt, WGs of the shrimp exposed to Na/K ratio 34.1 and 47.3 were significantly higher than those exposed to Na/K ratio 25.6 and 102.1 ( $P < 0.05$ ), and WG of the shrimp at Na/K ratio 153.3 was even lower ( $P < 0.05$ ). However, no significant differences were in WG among Na/K ratios at salinity 15 ppt ( $P > 0.05$ ), and the mean WG for shrimp at salinity 15 ppt was significantly higher than for that at salinity 30 ppt ( $P > 0.05$ ). SGR has the similar tendency as WG.

### Food Intake, Nutrient Retention and Food Conversion Efficiency

Data of food intake, nutrient retention and food conversion efficiency are presented in Table 3. No significant interaction effects were observed in feeding rates (FR) ( $P > 0.05$ ), though salinity and Na/K ratio both showed an effect ( $P < 0.05$ ). Shrimp at salinity 15 ppt had higher FRs than at salinity 30 ppt ( $P < 0.05$ ). Although no significant difference in FR was found among Na/K ratios at salinity 15 ppt ( $P > 0.05$ ), FR for shrimp exposed to Na/K ratio 25.6 showed the highest FR at salinity 30 ppt, which was significantly higher than for those exposed to Na/K ratio 34.1, 47.3 and 153.3 ( $P < 0.05$ ).

Protein retention (PR) and energy retention (ER) were significantly affected by Na/K ratio and interaction effects ( $P < 0.05$ ), whereas salinity showed no significant influence ( $P > 0.05$ ). At salinity 30 ppt, PR of the shrimp at Na/K ratio 153.3 was significantly lower than of those at the other 4 Na/K ratios ( $P < 0.05$ ), and no significant differences in PR were found between the latter 4 treatments ( $P > 0.05$ ). At salinity 15 ppt, significant differences in

TABLE 2.

Growth, survival and molting of *L. vannamei* in the artificial seawater of different salinities and Na/K ratios during 30 days.<sup>‡</sup>

Salinity (ppt)	Na/K ratio	Body wet weight (g)		WG (%)	SGR (% · day <sup>-1</sup> )	MF (% · day <sup>-1</sup> )	Survival (%)
		Initial	Final				
30	25.6	1.566 ± 0.010	5.289 ± 0.138 <sup>b</sup>	237.91 ± 10.65 <sup>b</sup>	4.05 ± 0.11 <sup>b</sup>	12.29 ± 0.31 <sup>ab</sup>	87.5 ± 7.2
	34.1	1.544 ± 0.006	6.277 ± 0.071 <sup>b</sup>	306.60 ± 4.87 <sup>c</sup>	4.67 ± 0.04 <sup>c</sup>	11.88 ± 1.20 <sup>ab</sup>	100.0 ± 0.0
	47.3	1.544 ± 0.017	6.198 ± 0.507 <sup>b</sup>	300.76 ± 29.71 <sup>c</sup>	4.60 ± 0.25 <sup>c</sup>	11.04 ± 0.63 <sup>a</sup>	100.0 ± 0.0
	102.1	1.566 ± 0.017	5.274 ± 0.135 <sup>b</sup>	236.82 ± 9.35 <sup>b</sup>	4.04 ± 0.09 <sup>b</sup>	14.38 ± 0.21 <sup>b</sup>	100.0 ± 0.0
	153.3	1.541 ± 0.009	3.491 ± 0.180 <sup>a*</sup>	126.56 ± 11.54 <sup>a*</sup>	2.71 ± 0.17 <sup>a*</sup>	13.96 ± 0.40 <sup>b</sup>	100.0 ± 0.0
15	25.6	1.553 ± 0.009	5.880 ± 0.231	278.46 ± 13.91	4.43 ± 0.12	10.56 ± 1.53	93.8 ± 6.3
	34.1	1.565 ± 0.015	6.115 ± 0.268	291.27 ± 20.95	4.53 ± 0.17	11.18 ± 0.44	100.0 ± 0.0
	47.3	1.558 ± 0.010	6.345 ± 0.209	307.47 ± 15.45	4.68 ± 0.13	12.29 ± 1.04	100.0 ± 0.0
	102.1	1.556 ± 0.017	5.826 ± 0.282	275.15 ± 21.70	4.39 ± 0.21	13.54 ± 0.79	93.8 ± 6.3
	153.3	1.550 ± 0.008	5.630 ± 0.123 <sup>*</sup>	263.15 ± 6.61 <sup>*</sup>	4.30 ± 0.06 <sup>*</sup>	13.33 ± 0.59	100.0 ± 0.0
Two-way analysis of variance <sup>‡</sup>							
Salinity	—	—	S (<0.001)	S (<0.001)	S (<0.001)	NS (0.317)	NS (0.999)
Na/K ratio	—	—	S (<0.001)	S (<0.001)	S (<0.001)	S (0.005)	NS (0.061)
Salinity × (Na/K ratio)	—	—	S (0.001)	S (0.001)	S (<0.001)	NS (0.486)	NS (0.566)

WG, weight gain; SGR, specific growth rate; MF, molting frequency.

<sup>†</sup> Mean ± SE of four replicates. Means within a column and within each salinity level followed by different letters are significantly different (Student-Neumann-Keuls multiple comparison,  $P < 0.05$ ). Means at the same Na/K ratio level were compared between salinity 30 and 15 ppt, an (\*) followed the means indicates significant difference ( $P < 0.05$ ).

<sup>‡</sup> Decimal fraction within each bracket denotes the  $P$  value of two-way ANOVA. S, significant ( $P < 0.05$ ); NS, not significant ( $P > 0.05$ ).

TABLE 3.  
Feeding, nutrient retention, food conversion and protein utility of *L. vannamei* during the 30-day experiment.<sup>‡</sup>

Salinity (ppt)	Na/K ratio	FR (%)	PR (%)	ER (%)	FCE (%)	PER
30	25.6	18.68 ± 0.40 <sup>b</sup>	32.98 ± 4.48 <sup>b</sup>	33.30 ± 4.47 <sup>b</sup>	19.39 ± 0.70 <sup>b</sup>	1.52 ± 0.06 <sup>b</sup>
	34.1	16.35 ± 0.58 <sup>a</sup>	36.97 ± 0.89 <sup>b</sup>	37.85 ± 0.91 <sup>b*</sup>	24.76 ± 0.85 <sup>a*</sup>	1.94 ± 0.07 <sup>a*</sup>
	47.3	16.53 ± 0.19 <sup>a</sup>	36.41 ± 2.39 <sup>b</sup>	36.88 ± 2.37 <sup>b</sup>	24.06 ± 1.01 <sup>a</sup>	1.88 ± 0.08 <sup>a</sup>
	102.1	17.66 ± 0.30 <sup>ab</sup>	31.92 ± 0.88 <sup>b</sup>	32.10 ± 0.87 <sup>b</sup>	20.50 ± 0.58 <sup>b</sup>	1.60 ± 0.05 <sup>b</sup>
	153.3	16.25 ± 0.73 <sup>a</sup>	22.46 ± 1.06 <sup>a*</sup>	22.03 ± 1.02 <sup>a*</sup>	15.78 ± 0.34 <sup>a*</sup>	1.24 ± 0.03 <sup>a*</sup>
15	25.6	19.13 ± 0.56	29.40 ± 1.16 <sup>a</sup>	30.19 ± 1.17 <sup>a</sup>	20.33 ± 0.99	1.59 ± 0.08
	34.1	17.86 ± 0.17	33.74 ± 1.02 <sup>ab</sup>	33.84 ± 1.00 <sup>ab*</sup>	22.05 ± 0.54 <sup>*</sup>	1.73 ± 0.04 <sup>*</sup>
	47.3	17.80 ± 0.21	34.79 ± 1.06 <sup>b</sup>	34.89 ± 1.05 <sup>b</sup>	22.67 ± 0.69	1.78 ± 0.05
	102.1	18.50 ± 0.35	31.74 ± 1.34 <sup>ab</sup>	32.12 ± 1.32 <sup>ab</sup>	20.82 ± 1.06	1.63 ± 0.08
	153.3	18.40 ± 0.33	31.21 ± 0.69 <sup>ab*</sup>	31.34 ± 0.68 <sup>ab*</sup>	20.60 ± 0.44 <sup>*</sup>	1.61 ± 0.03 <sup>*</sup>
Two-way analysis of variance <sup>‡</sup>						
Salinity		S (<0.001)	NS (0.974)	NS (0.977)	NS (<0.422)	NS (0.934)
Na/K ratio		S (0.001)	S (<0.001)	S (<0.001)	S (<0.001)	S (<0.001)
Salinity × (Na/K ratio)		NS (0.335)	S (0.017)	S (0.009)	S (<0.001)	S (0.012)

FR, feeding rate; PR, protein retention; ER, energy retention; FCE, food conversion efficiency; PER, protein efficiency ratio.

<sup>‡</sup> Mean ± SE of four replicates. Means within a column and within each salinity level followed by different letters are significantly different (Student-Neumann-Keuls multiple comparison,  $P < 0.05$ ). Means at the same Na/K ratio level were compared between salinity 30 and 15 ppt, an (\*) following the means indicates significant difference ( $P > 0.05$ ).

<sup>‡</sup> Decimal fraction within each bracket denotes the  $P$  value of two-way ANOVA. S, significant ( $P < 0.05$ ); NS, not significant ( $P > 0.05$ ).

PR were only found between shrimp exposed to Na/K ratio 25.6 and 47.3 ( $P < 0.05$ ). ER had the similar tendency as PR (Table 3).

Salinity showed no significant effects on food conversion efficiency (FCE) and protein utilization (PER) ( $P > 0.05$ ). However, at salinity 30 ppt, FCE was significantly affected by Na/K ratios. The FCEs for shrimp exposed to Na/K ratio 34.1 and 47.3 were significantly higher than for those exposed to Na/K ratio 25.6 and 102.1 ( $P < 0.05$ ), and the FCE for shrimp at Na/K ratio 153.3 was even lower ( $P < 0.05$ ). However, no significant differences in shrimp FCE were found among different Na/K ratios at salinity 15 ppt ( $P > 0.05$ ). PER showed the similar tendency as FCE.

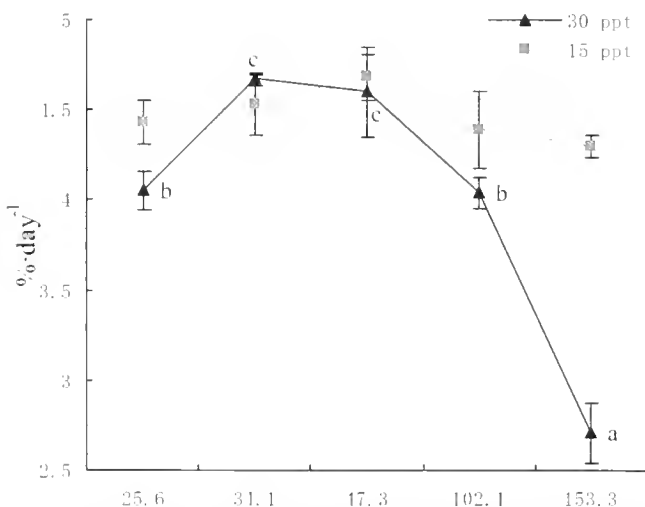


Figure 1. Specific growth rate of *L. vannamei* reared at different salinities and Na/K ratios for 30 days. Means ( $n = 4$ ), with letters significantly different within the same line ( $P < 0.05$ ). Bars represent standard errors of the means.

#### Interaction of Salinity and Na/K Ratio on Specific Growth Rate and Food Conversion Efficiency

As presented in Table 2 and Figure 1, significant effects of salinity, Na/K ratio and interaction were found in SGR ( $P < 0.05$ ). At both salinities SGR changed, whereas the Na/K ratio in seawater increased. However, the SGRs at salinity 30 ppt changed sharply, whereas those at salinity 15 ppt changed gently. At salinity 30 ppt, the SGR was low at Na/K ratio 25.6, but it mounted up rapidly at Na/K ratio 34.1 ( $P < 0.05$ ) and then declined a little at Na/K ratio 47.3 ( $P > 0.05$ ). Afterwards it made an acute drop at Na/K ratio 102.1 and 153.3 ( $P < 0.05$ ), respectively. Although no significant differences in SGR were found at salinity 15 ppt, it still could be observed that SGR rose gently, whereas Na/K ratio increased from 25.6–47.3 and then declined when Na/K ratio continued to increase. At salinity 30 ppt, the maximum value of SGR occurred at Na/K ratio 34.1, whereas at salinity 15 ppt, the maximum value emerged when Na/K ratio was 47.3, though the two values were almost equal.

Interaction effects on FCE are pictured in Figure 2. Similar to SGR, changes of FCE at salinity 30 ppt were much more acute than those at salinity 15 ppt. At salinity 30 ppt, shrimp got the highest FCE when Na/K ratio was 34.1, and at salinity 15 ppt they got it, whereas Na/K ratio was 47.3. However, under Na/K ratio 34.1 and 47.3, the FCEs at salinity 30 ppt were much higher than those at salinity 15 ppt. Otherwise, the FCEs at salinity 30 ppt were lower than those at salinity 15 ppt under the other Na/K ratios (25.6, 102.1 and 153.3). As a result, no significant differences were found between the mean values of FCEs at the two test salinities ( $P > 0.05$ ).

#### DISCUSSION

It is known that *L. vannamei* can tolerate a wide salinity range from brackish water of 1–2 ppt to hypersaline water of 50 ppt (Stern et al. 1990, McGraw et al. 2002). Boyd (1989) considered

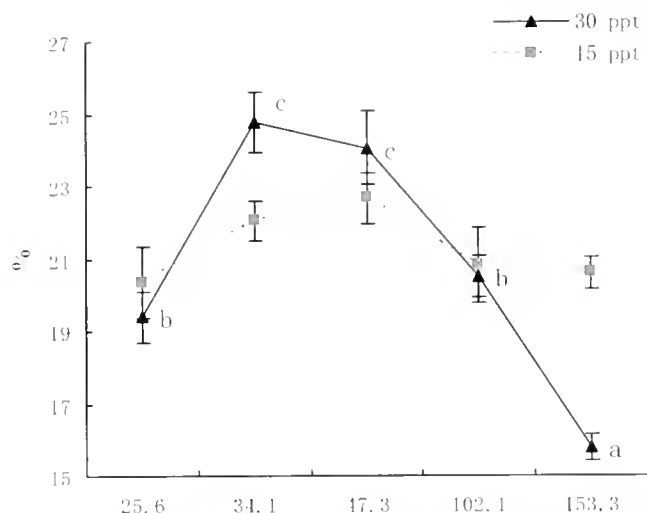


Figure 2. Food conversion efficiency of *L. vannamei* reared at different salinities and Na/K ratios for 30 days. Means ( $n = 4$ ), with letters significantly different within the same line ( $P < 0.05$ ). Bars represent standard errors of the means.

salinity of 15–25 ppt to be ideal for *L. vannamei* culture. But, in view of inconsistencies in published information regarding salinity effects on shrimp survival and growth, the optimum salinity for *L. vannamei* is still not conclusive. Bray et al. (1994) showed that 5 and 15 ppt treatments produced significantly greater final weights than other levels (25, 35 and 49 ppt) tested. However, Ponce-Palafox et al. (1997) concluded that the growth of *L. vannamei* was not reduced at the salinity range of 25–45 ppt, and there were significant interaction effects of salinity and temperature on the growth and survival rates of the shrimp. Laramore et al. (2001) found that the effects of salinity on growth varied with size/age. In this research, it is clear that interaction of salinity and Na/K ratio in seawater has significant influence on the growth of *L. vannamei* (Table 2). The mean final body weights, weight gains and specific growth rates of the shrimp at salinity 15 ppt were significantly higher than those at salinity 30 ppt ( $P < 0.05$ ), but it should be noted that good growth had always been obtained under Na/K ratio 34.1 and 47.3 at both salinities. At salinity 15 ppt, no significant effects were found in growth between treatments of different Na/K ratios ( $P > 0.05$ ), whereas the growth of shrimp under Na/K ratio 34.1 and 47.3 were much better than under the other ratios at salinity 30 ppt ( $P < 0.05$ ).

Customarily, salinity is one of the most compelling factors to most marine animals, but the ionic composition of saline water seems to be more important than salinity with regards to its effect on shrimp survival and growth (Davis et al. 2002, Saoud et al. 2003). Cawthorne et al. (1983) demonstrated that single salt solutions (NaCl) were not suitable for shrimp culture at any salinity. Additionally, Atwood et al. (2003) found that *L. vannamei* larvae could survive well in the solution containing 1 g/L sea salt, and they could survive fairly well after adding 4 g  $\text{CaCl}_2$  or 2 g  $\text{CaCl}_2$  and 2 g NaCl and the salinity reached 5 g/L, but no survival would have resulted if 4 g NaCl had been added. They speculated that sodium ratio to some other ion in the solution may be too high. Zhu et al. (2004) demonstrated that high Na/K ratio in seawater resulted poor survival of *L. vannamei*. In this experiment, the effects of salinity on growth, nutrient retention and food conver-

sion were much less than that of Na/K ratio and even interaction (Table 2, 3).

The importance of Na/K ratio might because of the indispensable role of potassium in crustacean osmolality maintenance and  $\text{Na}^+/\text{K}^+$  ATPase activity (Winkler 1986, Vargas-Albores & Ochoa 1992). It has been demonstrated in some penaeids that the sodium and/or potassium hemolymph concentrations were modified according to the concentration of these ions in the external medium (Castille & Lawrence 1981, Dall & Smith 1981). Potassium is important in the activation of  $\text{Na}^+/\text{K}^+$  ATPase (Mantel & Farmer 1983), which is involved in ion transport and osmoregulation. In the  $\text{K}^+$ -free saline, oxygen consumption of the excised gills of the shore crab *Carcinus mediterraneus* was reduced by almost 40% (Lucu & Pavicic, 1995), it suggested that 30% to 40% of the energy liberated by gill respiration is used by the branchial  $\text{Na}^+/\text{K}^+$  ATPase enzyme complex, maintaining  $\text{Na}^+$  and  $\text{K}^+$  concentration gradients between the extracellular and intracellular compartments. The central role of the  $\text{Na}^+/\text{K}^+$  ATPase in the crustacean gill epithelium and the sodium gradient that is used to drive numerous processed has been described (see Lucu 1990). The variation of salinity (ionic intensity) may directly affect the ionic gradients between the extracellular and intracellular compartments of the shrimp, which would inevitably impact the activity of  $\text{Na}^+/\text{K}^+$  ATPase, thus the interaction between salinity and Na/K ratio occurs.

Because of the limitation of labor and space, low salinity ( $\leq 5$  ppt) was not included in this experiment, and only 2 salinity levels (30 and 15 ppt) were tested, which made a flaw to the research. However, the result at 15 ppt might give some inspiration on the comprehension of effects of Na/K ratio at low salinity. Na/K ratios within the experimental range (25.6–153.3, mmol/mmol) showed little impact on the growth of *L. vannamei* at 15 ppt, which indicated that the shrimp were more adaptable to abnormal Na/K ratios at low salinity than at higher salinities. Additionally, a few recent literature have concerned the ionic challenge on the survival and growth of *L. vannamei* at low salinities and freshwater. McGraw and Scarpa (2003) demonstrated that necessity of potassium in “freshwater” (1 ppt) at a minimum concentration of 1 ppm for the survival of *L. vannamei* postlarval. It could be figured out that the 48-h survival of the shrimp did not differ significantly, whereas the Na/K ratio changed between 10 and 490 (mmol/mmol). Such results fairly coincided with the actions of the shrimp at salinity 15 ppt in the present study.

In conclusion, the interaction of salinity and Na/K ratio in seawater had significant effects on growth, feeding, nutrient retention and food conversion ( $P < 0.05$ ) of *L. vannamei*. The shrimp were more adaptable to abnormal Na/K ratios at low salinity than at higher salinities, and good growth could always be obtained within a Na/K range of 34.1–47.3 (mmol/mmol) regardless of salinity. However, further research was needed to confirm the proper range of Na/K ratio for the growth of shrimp at low salinities ( $\leq 5$  ppt).

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## INFLUENCE OF FEEDING ON HEPATOPANCREAS STRUCTURE AND DIGESTIVE ENZYME ACTIVITIES IN *PENAEUS MONODON*

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**ABSTRACT** This study examines the influence of feeding on digestive enzyme activities in the black tiger prawn, *Penaeus monodon*, using histological and biochemical techniques. Juvenile *P. monodon* (>50 mm total length) were sacrificed after feeding at sequential time intervals, with unfed controls also sacrificed at the same time intervals. Resin histology revealed that there were no morphological changes in the digestive gland F-cells between fed and unfed *P. monodon* over time. There were no significant changes in  $\alpha$ -amylase specific activities in fed animals over time post feeding, nor were there any changes in starved animals through time. Trypsin and  $\alpha$ -glucosidase activities were significantly higher in unfed animals than fed animals. There was a significant peak in  $\alpha$ -glucosidase activities at 0 min post feeding but this could not be conclusively determined as an influence from feeding because of high variability and the near negligible levels of  $\alpha$ -glucosidase activities. A peak in lipase activity was observed at 60 min post feeding, but because there was no significant differences in lipase activities between fed and unfed animals it was therefore inconclusive. Results from histological and biochemical analyses failed to prove that feeding had a significant influence on digestive enzyme production of *P. monodon* and suggest that digestive enzyme production in *P. monodon* may be continuous and is not strongly influenced by feeding.

**KEY WORDS:** shrimp, digestive enzymes, F-cells, feeding, hepatopancreas, lumen, *Penaeus monodon*

### INTRODUCTION

The most widely cultured prawn within the commercial prawn farming industry is the black tiger prawn, *Penaeus monodon*, because it has rapid growth rates and is larger than other farmed prawns at harvest. On such farms, food is often the highest operational cost (Chanratchakool et al. 1998). To be cost effective, prawn farms must achieve and maintain high feeding efficiencies while lowering food wastage. These requirements have paved the way for research into feeding strategies and penaeid enzyme studies, amidst other husbandry and nutrition studies.

Knowledge of penaeid enzyme dynamics, which is biologically important for the culture of these animals, has only been provided by generalist observations between feeding and/or feeding behavior in relation to digestive and cellular processes, or by direct histological examination. A continuous digestive enzymatic activity allows maximal digestive efficiency at all times but incurs a higher energy cost. Conversely, an induced response of increased enzyme secretion after food ingestion allows the organism to put more energy into somatic and/or gonadal growth because energy is not largely spent on continuous enzyme production. By clarifying the physiological aspects of feeding on the digestive functions, improved management of the cultured animals may be achieved.

Digestion in penaeids is facilitated by the hepatopancreas, which has several key roles including secretion of digestive enzymes, digestion and absorption of nutrients, storage of reserves and disposal of waste products (Al-Mohanna et al. 1985a, Al-Mohanna et al. 1985b, Al-Mohanna & Nott 1987, Vogt 1993). It is bilobed and comprises numerous blind-ending tubules (Gibson & Barker 1979, Dall 1992, Brunet et al. 1994, Vogt 1994). These tubules are lined by epithelial cells, which are differentiated into 4 distinct types: E- (embryonic), F- (fibrillar), B- (blister-like) and R- (resorptive) cells (Jacobs, 1928). F-cells are involved in enzyme secretion. Al-Mohanna et al. (1985a), observed vesicles budding from Golgi bodies in the F-cells of fed green tiger prawn *Penaeus*

*semisulcatus* and suggested that these vesicles were enzyme precursors or zymogens, but this is yet to be confirmed or observed in other penaeids (Dall 1992, Icely & Nott 1992). Vesicles were also observed in F-cells of the crayfish *Astacus astacus* after artificial draining of gastric juice (Vogt et al. 1989, Vogt 1996). The presence of digestive enzymes within these vesicles has been demonstrated by several authors with the use of immunohistochemistry (Malcoste et al. 1983), immunofluorescence (Vogt et al. 1989) and in situ hybridization (Van Wormhoudt et al. 1995).

The direct influence of feeding on enzyme production has been demonstrated in the European lobster *Homarus gammarus* by Barker and Gibson (1977). Using histology, they observed the discharge of supranuclear contents (digestive enzymes) from secretory cells after ingestion. Barker and Gibson (1978) found a similar trend from an identical experiment on the mud crab *Scylla serrata*. Similar results were also observed by Al-Mohanna et al. (1985a) in *P. Semisulcatus*, with the appearance of numerous zymogen granules in the F-cells corresponding to a maximal rate of enzyme discharge into the tubule lumen.

This study aims to clarify the effects of feeding on digestive enzyme production on the commercially important prawn *P. monodon* using histological and biochemical methods. By applying both techniques, confirmation of the effects of feeding on the hepatopancreas will hopefully be possible. The specific aims of this study are to examine the dynamics of the digestive gland in response to a single feeding event, by documenting changes in the structure of the hepatopancreas and the morphology of the F-cells and the specific activity of digestive enzymes. This was facilitated using a time series post feeding analysis on *P. monodon* juveniles.

### MATERIALS AND METHODS

#### *Animals and Husbandry*

*P. monodon* post larvae were sourced from Rocky Point Prawn Farm, Queensland, Australia. The animals were housed in three 800-L tanks on a recirculation system and were fed daily to satiation with *Artemia* and commercial prawn pellets (Grobest t4, Primo Aquaculture).

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### Experimental Design and Procedure

The animals used in the experiment ( $TL > 5$  cm) were randomly caught from the stock population using dipnets. Molt stage was determined by examination of setogenesis via changes in the seta on the margin of the inner uropod, in accordance with Smith and Dall (1985). Intermolt animals were randomly stocked into individual 1L containers to ensure that animals had equal access to feed and to prevent feeding hierarchies and cannibalism among conspecifics (Heinen 1987). The containers were then randomly allocated in the three holding tanks and kept afloat with Styrofoam sheets. The animals were placed under constant light to ensure that any enzymatic response was a function of feeding and not influenced by diurnal cycles. They were starved for 48 h to allow the animals to acclimatize to the containers as well as to ensure that stomach contents were emptied and that there would not be any residual effect from prior feeding regimes that would influence the results.

Fed animals were allowed to feed on commercial prawn pellets (Grobest T4, Primo Aquaculture) for 20 min. This feeding event was timed from the moment the animals collected the pellets with their periopods. At the end of 20 min, uneaten food was removed with a siphon. Unfed animals were disturbed in a similar fashion and were used as a control. At each of the times outlined in Table 1, five fed and five unfed animals were removed from their containers and placed in an ice slurry for 10 min to induce a chill coma.

The prawns were weighed and the hepatopancreas removed. Half the hepatopancreas was snap frozen in liquid Nitrogen and stored at  $-80^{\circ}\text{C}$  for later digestive enzyme analysis. The remaining tissue was used for histology.

### Wax Histology

One quarter of each hepatopancreas was fixed in Bouin fixative and processed routinely for wax histology. Sections ( $5\ \mu\text{m}$ ) were cut using a Microm HM 340 microtome and stained with Hematoxylin and Eosin for structural examination or with Mercuric Bromophenol Blue to elucidate the location of proteins (Chapman 1975). Sections were examined at  $\times 100$  magnification with an Olympus BH-2 light microscope. Images were scanned with a microscope-mounted Leica DC 300F digital camera and examined on an IBM computer using an image manager, Leica IM50. To determine if tubule lumen size changed in response to feeding over time, five tubules per slide were randomly selected using a numbered grid and a random number table. The area of the lumen and

the tubule were measured using the freehand function of the image manager program.

### Resin Histology

Approximately  $2\ \text{mm}^3$  of hepatopancreas tissue from each animal was fixed in 5% glutaraldehyde in 2% sucrose-phosphate buffer pH 7.4 for 2–3 h, washed with 2% sucrose-phosphate buffer, dehydrated with ethanol and embedded in JB4 resin. Sections ( $2\ \mu\text{m}$ ) were then cut and stained with polychrome stain. Changes in cellular structure in response to feeding and elapsed time after feeding/disturbance were described with emphasis on F-cell morphology because they are the enzyme secreting cells (Al-Mohanna et al. 1985a, Vogt et al. 1989, Vogt 1996).

### Enzyme Extraction

Hepatopancreas tissue was thawed on ice and homogenized using an Ultra Turrax electric homogenizer (IKA-Werke, Germany) in 1 mL of chilled 0.1 M Tris 0.02M NaCl pH 7.0 buffer for 1 min. The homogenate was centrifuged at 10,000 rpm for 5 min to pellet debris and solids and the supernatant (subsequently referred as enzyme extract) stored at  $-20^{\circ}\text{C}$ .

### Enzyme Assays

One enzyme unit was defined as the amount of enzyme that catalyzed the release of 1  $\mu\text{mole}$  of product per minute and was calculated using the appropriate molar extinction coefficient ( $\epsilon$ ) in the assay conditions or a standard curve. Specific activity was defined as enzyme activity per mg of protein ( $\text{Units mg protein}^{-1}$ ). Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as the standard. Spectrophotometric enzyme assays (200  $\mu\text{L}$  microassays) were performed in duplicate at  $37^{\circ}\text{C}$  in IWAKI flatbottom microplates and absorbances read in a Tecan Spectro Rainbow Thermo microplate reader. Appropriate controls were included with each analysis. Tests confirmed that enzyme activities were linear with incubation time.

### Trypsin

Trypsin activity was measured using N- $\alpha$ -benzoylarginine-p-nitroanilide (BAPnA) (Sigma B-4875) as the substrate. Each 200- $\mu\text{L}$  assay contained 180- $\mu\text{L}$  0.2 M Tris 0.2 M NaCl pH 7.5 buffer, 10  $\mu\text{L}$  BAPnA dissolved in dimethylformamide (DMF) and 10  $\mu\text{L}$  enzyme extract. Trypsin activity was determined by measuring the release of p-nitroanilide at  $A_{405\text{nm}}$  and using the molar absorption coefficient for p-nitroanilide,  $9,300\ \text{m}^{-1}\ \text{cm}^{-1}$  (Stone et al. 1991).

### $\alpha$ -Amylase

$\alpha$ -Amylase activity was measured using Ethylidene-pNP-G7 (E- pNP-G7) as the substrate. Each 200  $\mu\text{L}$  assay contained 195  $\mu\text{L}$  of Infinity amylase reagent (Sigma 568–20) and 5  $\mu\text{L}$  enzyme extract (thawed on ice) at  $37^{\circ}\text{C}$ .  $\alpha$ -Amylase activity was determined by measuring the release of p-nitrophenol at  $A_{405\text{nm}}$  and using the molar absorption coefficient for p-nitrophenol,  $10\ 130\ \text{m}^{-1}\ \text{cm}^{-1}$ .

### $\alpha$ -Glucosidase

$\alpha$ -Glucosidase activity was measured using p-nitrophenol  $\alpha$ -D-glucopyranoside (Sigma N-1377) as the substrate. Each assay, performed in a  $37^{\circ}\text{C}$  water bath, contained 350  $\mu\text{L}$  0.1 M Tris 0.02 M

TABLE 1.  
Experimental times for feeding/disturbance and sacrifice.

Time	Time When Fed Animals Were Sampled	Time When Unfed Animals Were Sampled
0 min	Immediately after feeding event	Immediately after disturbance
30 min	30 min after feeding event	30 min after disturbance
60 min	1 h after feeding event	1 h after disturbance
120 min	2 h after feeding event	2 h after disturbance
240 min	4 h after feeding event	4 h after disturbance
480 min	8 h after feeding event	8 h after disturbance

NaCl pH 7 buffer, 100  $\mu\text{L}$  0.02 M p-nitrophenol  $\alpha$ -D-glucopyranoside and 50  $\mu\text{L}$  enzyme extract. Aliquots (20  $\mu\text{L}$ ) of the assay mixture were removed at 1 min and 40 min after addition of the enzyme extract and added to 180  $\mu\text{L}$  1 M  $\text{Na}_2\text{CO}_3$  to stop the enzyme reaction.  $\alpha$ -Glucosidase activity was determined by release of p-nitrophenol at  $A_{400\text{nm}}$  and using the molar absorption coefficient for p-nitrophenol,  $18\,300\text{ m}^{-1}\text{ cm}^{-1}$  (Erlanger et al. 1961).

#### Lipase

Lipase activity was measured using 4-p-nitrophenol caproate (4NPC) (SigmaN-0502) as the substrate. Each 200  $\mu\text{L}$  assay contained 190  $\mu\text{L}$  0.5M Tris 0.1 M NaCl pH 8.5 buffer, 10  $\mu\text{L}$  of 2.5 mM 4NPC in ethanol and 10- $\mu\text{L}$  enzyme extract (thawed on ice) at 37  $^{\circ}\text{C}$ . Lipase activity was determined by measuring by the release of p-nitrophenol at  $A_{405\text{nm}}$  and using the molar absorption coefficient for p-nitrophenol,  $19\,800\text{ m}^{-1}\text{ cm}^{-1}$  (Gjellesvik et al. 1992). A blank assay containing only 195  $\mu\text{L}$  buffer and 5  $\mu\text{L}$  substrate was also performed for each sample to deduct background absorbance from actual enzyme activity measurements.

#### Statistical Analysis

Chi-square tests of independence were used to determine if the area of tubule lumen changed as a function of feeding over time. The occurrence of autolysis in some animals resulted in insufficient replicates for statistical comparison ( $n < 3$ ) of hepatopancreas structure from the sampling times of 120 min and 480 min post feeding. As a result, only the remaining 4 sampling times (0 min, 30 min, 60 min and 480 min post feeding) were analyzed from the histology. Orthogonal analyses of variance (ANOVA) were used to determine the effect of feeding on production of digestive enzymes. Homogeneity of variances was assessed using residual plots, and data were transformed with a square root transformation when the assumption of homogeneity of variances was violated.

## RESULTS

#### Structure of the Hepatopancreas

The penaeid hepatopancreas consisted of numerous blind-ending tubules, each made up of a number of epithelial cell types. The epithelial cells rest on a basement membrane and surround a central lumen (Fig. 1A). F-cells stained positively in a darker blue with Mercuric Bromophenol Blue caused by the presence of proteins (Fig. 1B). The other cell types stained light blue except for the nucleus, where nuclear material was dark blue (Fig. 1B). Greater structural detail revealed from resin histology showed F-cells also had pink patches within the cytoplasm but their composition could not be determined from the polychrome stain (Fig. 1C).

There were no apparent differences in the morphology of the F-cells between the fed and unfed animals, nor were there any structural changes over time post feeding. There were also no zymogen granules or digestive vesicles observed.

The area of lumen did not change significantly over time post feeding ( $F = 2.703$ ,  $\text{df } 3, 23$ ,  $P = 0.069$ ). It was also not significantly different between fed and unfed animals ( $F = 2.448$ ,  $\text{df } 1, 23$ ,  $P = 0.131$ ). The mean lumen area was  $37.8 \pm 6.7\%$  of the tubule area.

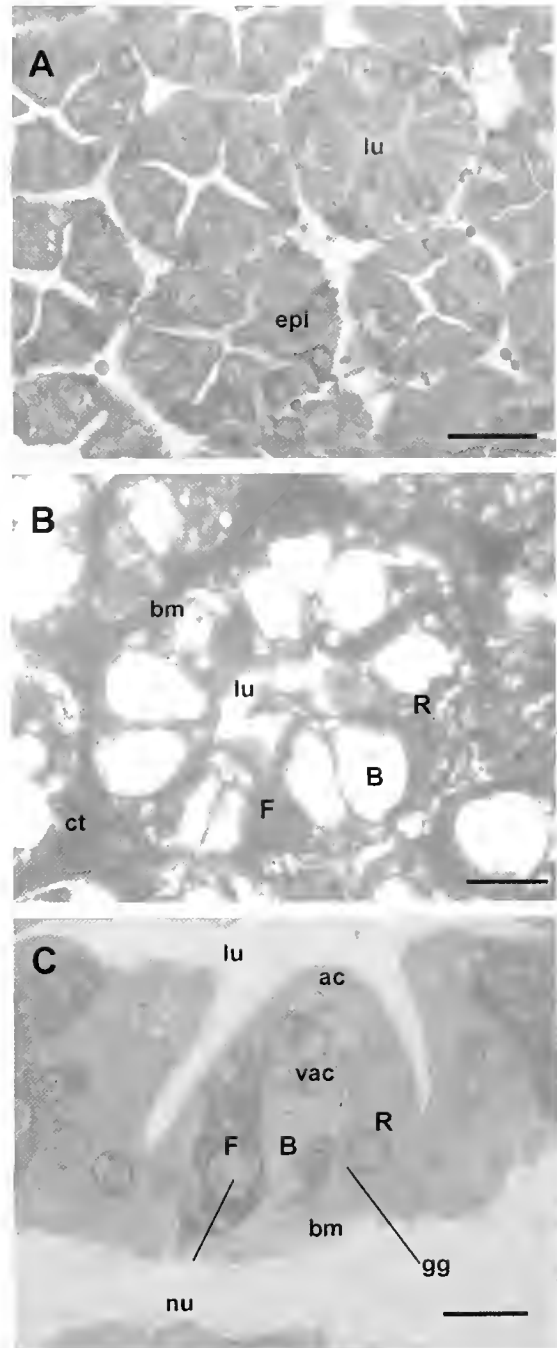


Figure 1. Transverse resin and wax sections of *P. monodon* hepatopancreas tubules. A. Hepatopancreas stained with polychrome stain. Scale bar, 50  $\mu\text{m}$ . B. Hepatopancreas stained with Mercuric Bromophenol Blue. Scale bar, 25  $\mu\text{m}$ . C. Cluster of hepatopancreas epithelial cells stained with polychrome stain. Scale bar, 20  $\mu\text{m}$ . F, F-cells; B, B-cells; R, R-cells; ac, apical complex; bm, basement membrane; ct, connective tissue; epi, tubule epithelial cells; gg, glycogen; lu, lumen; nu, nucleus; vac, central vacuole.

#### Digestive Enzymes

All of the enzymes tested were present in the hepatopancreas of the fed and unfed animals (Table 2), although activities were variable between individuals. The orthogonal ANOVA revealed that the interaction between enzyme dynamics, effect of feeding and

TABLE 2.

The mean specific activities ( $\mu\text{mol min}^{-1} \text{mg protein}^{-1}$ ) of the enzymes from the hepatopancreas of fed and unfed *P. monodon* juveniles, with standard error and coefficient of variation.

	Trypsin	$\alpha$ -Amylase	$\alpha$ -Glucosidase	Lipase
Fed				
mean $\pm$ SE	2.61 $\pm$ 0.64	18.41 $\pm$ 1.9	0.6 $\times 10^{-3} \pm 4.88 \times 10^{-8}$	0.39 $\pm$ 0.03
CV	1.27	0.54	3.9 $\times 10^{-4}$	0.38
Unfed				
mean $\pm$ SE	5.06 $\pm$ 1.84	25.48 $\pm$ 26.72	1.4 $\times 10^{-3} \pm 2.8 \times 10^{-8}$	0.37 $\pm$ 0.01
CV	1.92	5.55	1.04 $\times 10^{-3}$	0.18

elapsed time post feeding was not significant (Table 3). Therefore fed and unfed animals were grouped together when examining enzyme dynamics over time, and vice versa.

### Trypsin

Trypsin specific activity in the unfed animals was twice as high as that of fed animals (Table 2, 3). There were no significant differences in trypsin specific activity over time post feeding/disturbance (Table 3).

### $\alpha$ -Amylase

There were no significant differences in  $\alpha$ -amylase specific activity between fed and unfed animals (Table 2). In addition,  $\alpha$ -amylase specific activity did not change significantly over time post feeding/disturbance (Table 3).

### $\alpha$ -Glucosidase

Although  $\alpha$ -glucosidase specific activities in both fed and unfed animals were low, the specific activity of  $\alpha$ -glucosidase in the unfed animals was 3-fold higher than that of fed animals (Table 2).  $\alpha$ -Glucosidase specific activity also significantly differed over time (Table 2). Specific activity at 0 min was approximately four times higher than the activity at 240 min post feeding/disturbance (Fig. 2).

### Lipase

There was no difference in lipase specific activity between fed and unfed animals (Table 2). However, lipase activity differed significantly over time (Table 2). Lipase activity remained relatively constant at 0 min and 30 min then peaked at 60 min after which the activity declined significantly through to 480 min post feeding/disturbance. Lipase activity at 60 min post feeding was twice the activity than at 480 min post feeding/disturbance (Fig. 3).

## DISCUSSION

This study is the first to examine the effect of feeding on digestive enzyme production in the commercially important black tiger prawn *P. monodon* using the combination of sensitive techniques, resin histology and spectrophotometric biochemical assays. These methods yield quantitatively more robust results than enzyme histochemical visualization used by Barker and Gibson (1977), histological analysis on F-cell differentiation after feeding via chemical tracers used by Al-Mohanna et al. (1985a) and immunohistochemistry with radiochemical tracers by Vogt et al. (1989). Most of previous research on digestive enzyme activities, as listed in Tables 4 and 5, were not used for analyzing the effect of feeding on digestive enzyme production, but were performed on animals that were fed constantly prior to analysis.

Furthermore, the short 20 min feeding period in this experiment was used to initiate a feeding response and associated digestive enzyme production and to allow for sampling at 30 min post feeding/disturbance (Table 1). A longer feeding period or the complete filling of the animals' gut was not necessary for the commencement of enzyme production. The 48-h starvation period for the animals was also implemented to ensure an empty gut prior to commencement of the experiment. This starvation period was sufficient and similar to the duration used by Al-Mohanna et al. (1985a) on *P. semisulcatus*. A week-long starvation period used on *A. astacus* by Vogt et al. (1989) was considered too long for a smaller tropical decapod like *P. monodon*.

The histological and biochemical results from this study suggest that *P. monodon* has a continuous digestive enzyme production, regardless of feeding, and contradicts previous studies that suggested an influence of feeding on enzyme production.

### Effect of Feeding on F-cell Structure

The positive reaction of F-cells to Mercuric Bromophenol Blue confirms their role in digestive enzyme production. The negative reaction by B- and R-cells to Mercuric Bromophenol Blue also

TABLE 3.

Interactions between and among factors for the 4 examined enzymes.

Enzyme	Interaction (Time $\times$ Treatment)	Time	Fed Versus Unfed
Trypsin	F = 0.592, df 5, 43, <i>P</i> = 0.706	F = 0.409, df 5, 43, <i>P</i> = 0.84	F = 11.741, df 1, 43, <i>P</i> = 0.001
$\alpha$ -Amylase	F = 0.799, df 5, 43, <i>P</i> = 0.557	F = 0.376, df 5, 43, <i>P</i> = 0.863	F = 3.884, df 1, 43, <i>P</i> = 0.055
$\alpha$ -Glucosidase	F = 0.417, df 5, 43, <i>P</i> = 0.834	F = 2.853, df 5, 43, <i>P</i> = 0.026	F = 9.998, df 1, 43, <i>P</i> = 0.003
Lipase	F = 0.462, df 5, 43, <i>P</i> = 0.802	F = 3.21, df 5, 43, <i>P</i> = 0.015	F = 0.08, df 1, 43, <i>P</i> = 0.927

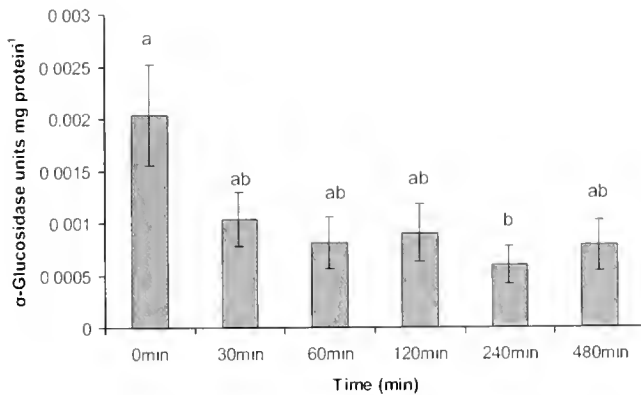


Figure 2. Changes in activity of  $\alpha$ -glucosidase in the hepatopancreas of *P. monodon* juveniles over time after a feeding event/disturbance. Means with different letters denote significant differences ( $P < 0.05$ ).

confirms that these cells do not function in enzyme secretion. These findings are consistent with several other studies (Dall & Moriarty 1983, Al-Mohanna et al. 1985a). Resin histology revealed pink patches within the cytoplasm of F-cells in both fed and unfed animals, but the nature of the organelles could not be conclusively determined from the polychrome stain (Fig. 1C). However, it is likely that these pink patches were rough endoplasmic reticulum (rER) or Golgi bodies, based on comparisons with previous studies on F-cell structure (Dall & Moriarty 1983; Al-Mohanna et al. 1985a). These structures could be better identified with electron microscopy.

Examination of fed and unfed animals post feeding in this study revealed a lack of structural differences in F-cells of both fed and unfed animals post feeding/disturbance. There was no evidence to suggest that F-cells of *P. monodon* had a distinct response to feeding through either digestive enzyme production via formation of zymogen granules (Al-Mohanna et al. 1985a) or appearance of vesicles (Vogt et al. 1989). This differs from several studies where histology demonstrated the influence of feeding on enzyme production.

Barker and Gibson (1977, 1978) demonstrated a positive response of enzyme production post feeding in the F-cells of the European lobster *Homarus gammarus* and the mud crab *Scylla Ser-rata* within an hour after feeding. They observed three bursts of digestive enzyme discharge over a period of 5 h post feeding from the F-cells in both species, with each burst of activity measured by visual assessment of the intensity of the colored reaction product

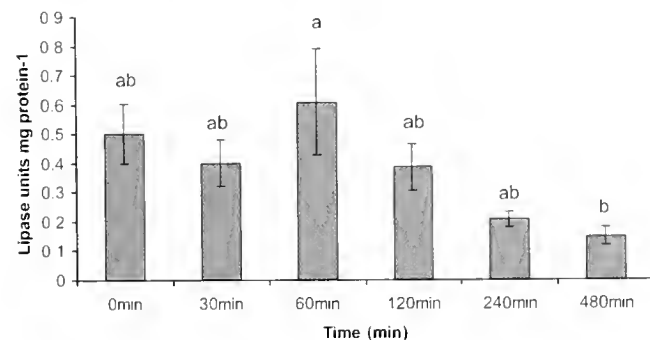


Figure 3. Changes in activity of lipase in the hepatopancreas of *P. monodon* juveniles over time after a feeding event/disturbance. Means with different letters denote significant differences ( $P < 0.05$ ).

from histochemical sections. Al-Mohanna et al. (1985a) also reported a positive response of enzyme production post feeding in the F-cells of *P. semisulcatus* using electron microscopy, with one occurrence of enzyme production in the form of zymogen granules. However, it should be noted that no other authors have found zymogen granules in penaeids (Dall 1992, Icely & Nott 1992). Vogt et al. (1989) suggested that feeding had an influence on enzyme production, because enzyme vacuoles were observed in F-cells in fed crayfish *Astacus astacus*. The size of zymogen granules reported by Al-Mohanna et al. (1985a) were approximately that of Golgi bodies in F-cells and enzyme vesicles observed by Vogt et al. (1989) were similar in size to adjacent mitochondria. Although electron microscopy was not performed in the current study, F-cell organelles like Golgi bodies and mitochondria could be distinguished in resin sections. However, there was no evidence of zymogen granules or enzyme vesicles in the F-cells of *P. monodon*.

Furthermore, although these histological studies suggest that feeding influenced enzyme secretion, there is ambiguity in their conclusions because no starved controls were used. Thus the authors did not definitively demonstrate the influence of feeding on enzyme production, therefore it is possible that disturbance caused by feeding may be contributing to the observed changes and needs to be confirmed with the use of starved controls.

#### Analysis of Lumen Area After Feeding

During digestion, digestive enzyme secretions flow from the F-cells of the digestive gland into the tubule lumen and ultimately into the primary duct and then the foregut. Chyme (digestive fluid from the foregut) also migrates from the foregut to the hepatopancreas via the same route. It has been suggested that as the prawns feed, the hepatopancreas tubules become loaded with chyme and fine particles masticated by the gastric mill (Al-Mohanna & Nott 1987). However, there have not been any studies to elucidate changes in the size of the tubule lumen as a response to feeding.

Based on suggestions that the hepatopancreas tubules would be filled with digestive enzymes and chyme during digestion, it was believed that the cross-sectional area of the lumen, as a percentage of tubule area, would expand and contract in response to the movement of fluids. However, this study shows that there was no significant change in lumen area, which suggests that the fluid flow (enzyme secretions or chyme) within the lumen of the tubules was either constant, or the rate of flow was not strong enough to cause a significant change in lumen area. A constant fluid flow within the tubule lumen is indicative that enzyme production was likely to be continual.

#### Digestive Enzymes of Fed Versus Unfed Animals Over Time

It has been reported that feeding triggers enzyme production in decapods (Barker & Gibson 1977, Barker & Gibson 1978, Al-Mohanna et al. 1985a, Vogt et al. 1989), as shown by higher enzyme activities in the fed animals over time. However, these authors did not conclusively demonstrate the influence of feeding on enzyme production, because starved controls were not used, so it is possible the enzymatic response may be because of disturbance at feeding rather than the feeding event itself. In this study, we did not find any evidence from the enzyme analyses to conclude that *P. monodon* also displayed a positive digestive response to feeding.

We found that there was no significant differences in amylase

activity between fed and unfed animals, or alternatively that trypsin and  $\alpha$ -glucosidase activities in the fed animals were significantly lower than those in the unfed animals. The increased trypsin and  $\alpha$ -glucosidase activity in unfed animals could be because of starvation. Prawns have been shown to use carbohydrate, lipid and protein reserves sequentially during starvation (Cuzon et al. 1980), which may be depleted after approximately 2 days of starvation (Stuck et al. 1996). Therefore, it is possible that the extended starvation period imposed on the unfed animals during the experiment's preparation in this study could thus have caused the increase in trypsin and  $\alpha$ -glucosidase activity.

Hernandez-Cortes et al. (1999) also found no significant difference in trypsin activity between fed and unfed *P. vannamei*. This lack of significant difference in trypsin activity as demonstrated by Hernandez-Cortes et al. (1999), supports our findings that feeding did not have an influence on enzyme production, which would be indicated by an increase of trypsin in fed animals.

In this first examination of lipase activities in *P. monodon*, it was found that lipase activity peaked at 60 min post feeding, which was twice the activity at 480 min post feeding (Fig. 3). There was also a decreasing trend of enzyme activities over time observed in the  $\alpha$ -glucosidase assay (Fig. 2). Both these trends are common for fed and unfed animals, because of the lack of significance in the orthogonal ANOVA, and they suggest that this trend may be caused by disturbance and not actual provision of feed.

#### Comparison of Examined *P. monodon* Enzyme Activities With Other Decapods

The trypsin activities in *P. monodon* were similar to reported activities in most penaeids (Table 4). The higher trypsin activity in *C. maenus* is probably caused by its mainly carnivorous diet. Trypsin activity is influenced by the amount of protein and protein source in the diet. This was demonstrated by Lee et al. (1984), who found that *P. vannamei* fed a diet with a 38% protein inclusion gave a significantly higher protease activity. Smith et al. (1985), found similar results with *P. vannamei* when the diet used had a 36% protein inclusion. Because the amount of protein in the commercial prawn pellets used in this study (39%) was similar to the protein inclusions in the diets used by Smith et al. (1985) and Lee et al. (1984), it could be assumed that the trypsin activity generated by *P. monodon* in this study was efficiently assimilating the protein in the diet.

Smith et al. (1985) also found that the protein source was more substantial than protein amount on influencing growth in medium and large animals (mean weights 9.8 g and 20.8 g respectively). Rodriguez et al. (1994) made a similar suggestion that trypsin activity could be influenced by diet. In their study, *P. japonicus* feeding on the algae *Chaetoceros gracilis* had six times more trypsin than animals feeding on *Artemia nauplii* (Rodriguez et al. 1994). Thus, the type of proteins in the diet must also influence the

TABLE 4.

Comparison of trypsin and  $\alpha$ -amylase activities between *Penaeus monodon* (fed and unfed) and other penaeids. Animals were in the intermolt phase and were postlarval or juveniles. The units were reported in units  $\text{mg}^{-1}$  protein, where units are  $\mu\text{mol min}^{-1}$ .

Species	Trypsin		$\alpha$ -Amylase		References
	Activity	Method	Activity	Method	
<i>P. monodon</i> (fed)	2.61	Spectrophotometric assay, N- $\alpha$ -benzoylarginine-p-nitroanilide	18.41	Spectrophotometric assay, Ethylidene-pNP-G7	This study
<i>P. monodon</i> (unfed)	5.06	Spectrophotometric assay, N- $\alpha$ -benzoylarginine-p-nitroanilide	25.48	Spectrophotometric assay, Ethylidene-pNP-G7	
<i>P. californiensis</i>			16*	Spectrophotometric assay, 1% starch	Vega-Villasante et al. 1993
<i>P. indicus</i>	0.62	Spectrophotometric assay, $\alpha$ -N-benzoyl-L-arginine-p-nitroanilide			Honjo et al. 1990
<i>P. indicus</i>			0.5*	Spectrophotometric assay, 1% starch	Omondi and Stark, 1995
<i>P. japonicus</i>	12*	Spectrophotometric assay, N- $\alpha$ -p-toluenesulphonyl-L-arginine methyl ester	8*	Spectrophotometric assay, 1% w/v starch	Rodriguez et al. 1994
<i>P. mulleri</i>	0.9	Spectrophotometric assay, N-benzoyl-DL-arginine-p-nitroanilide			Fernandez Gimenez et al. 2001
<i>P. vannamei</i>	0.072	Spectrophotometric assay, benzoyl-DL-arginine-p-nitroanilide			Lee et al. 1984
<i>P. vannamei</i>			0.15*	Spectrophotometric assay, 1% starch	Omondi and Stark, 1995
<i>Litopenaeus vannamei</i>	9.3	Spectrophotometric assay, N- $\alpha$ -p-toluenesulphonyl-L-arginine methyl ester			Puello-Cruz et al. 2002

\* Denotes figures that were estimated from values reported in graphs.

trypsin activity. Because trypsin activity has been found in *P. monodon* in this study, it could also be safely assumed that other proteases like carboxypeptidases were present in *P. monodon* to complete protein digestion. These other enzymes, although not examined, were found to be present in other penaeids (Lee et al. 1984).

The  $\alpha$ -amylase activity in *P. monodon* differed from the reported  $\alpha$ -amylase activities in other penaeids (Table 4) and could be explained by different experimental treatments as well as genetic differences between species.

$\alpha$ -Glucosidase is necessary for the final liberation of glucose residues from oligosaccharides that have been formed from the hydrolysis of large carbohydrates by  $\alpha$ -amylase. The low activity of  $\alpha$ -glucosidase from *P. monodon* studied here was lower than reported activities (Table 5), but was likely to be sufficient for complete carbohydrate hydrolysis. It seems that penaeids have naturally low  $\alpha$ -glucosidase activities, because nearly negligible  $\alpha$ -glucosidase activities were also found in *P. indicus* and *P. vannamei* (Omondi & Stark 1995, Le Chevalier & Van Wormhoudt 1998).

Past research has also shown a crustaceans' ability to digest carbohydrates varies with the type of carbohydrates provided in the diet (Van Wormhoudt & Favrel 1988). Generally better growth has been attained in crustaceans with the use of complex carbohydrates than with simple mono or disaccharides (Van Wormhoudt & Favrel 1988). Because there is a wide range of carbohydrates found in naturally occurring food (Kristensen 1972), it seems appropriate that prawns would exhibit a diverse carbohydrase profile to exploit the range of dietary carbohydrates (Wigglesworth & Griffith 1994). The carbohydrases examined in this study are just two of the many carbohydrases described by other authors (Wigglesworth & Griffith 1994).

Deering et al. (1996) examined lipase activity in *P. monodon* but were not able to quantify the lipase activity, as it was only demonstrated with the use of a triolein/agar emulsion screening

(Table 5). Lipase activity was also found in other penaeids as reviewed by Jones et al. (1997) but was expressed as percent occurrence. There is currently a limited amount of literature on lipase activity in penaeids. As indicated by Le Vay et al. (2001), there should be more studies on lipid hydrolysis because current focus has been on penaeid proteases.

## CONCLUSION

This study is the first to investigate the effect of feeding on the digestive enzyme production of decapods by using histological and quantitative enzymatic techniques. There were no trends that suggest feeding had an effect on the structure of the hepatopancreas or enzymatic activity. The morphological changes in the F-cells of other crustaceans examined by Barker and Gibson (1977, 1978), Al-Mohanna et al. (1985a) and Vogt et al. (1989) were not observed in this study. The lack of structural changes in the F-cells was further supported by the lack of conclusive results from the enzyme analyses. The commercial prawn feed used in this experiment contained all the necessary nutrients required by the prawns. Therefore it is expected that all the enzymes tested would be produced for proper digestion of the feed. Perhaps subsequent studies using monoingredient diets would be able to ascertain individual enzyme dynamics. In summary, the results from this study suggest a continuous enzyme production in *P. monodon*, which occurred even in the absence of food. These findings could be further confirmed by additional research with the use of continuously fed control animals.

## ACKNOWLEDGMENTS

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TABLE 5.

Comparison of  $\alpha$ -glucosidase and lipase activities between *Penaeus monodon* (fed and unfed) and other penaeids. Animals were in the intermolt phase and were postlarval or juveniles. The units were reported in units  $\text{mg}^{-1}$  protein, where units are  $\mu\text{mol min}^{-1}$ .

Species	$\alpha$ -Glucosidase		Lipase		References
	Activity	Method	Activity	Method	
<i>P. monodon</i> (fed)	$0.6 \times 10^{-3}$	Spectrophotometric assay, $\rho$ -nitrophenol $\alpha$ -D-glucopyranoside	0.39	Spectrophotometric assay, 4- $\rho$ -nitrophenol caproate	This study
<i>P. monodon</i> (unfed)	$1.4 \times 10^{-3}$	Spectrophotometric assay, $\rho$ -nitrophenol $\alpha$ -D-glucopyranoside	0.37	Spectrophotometric assay, 4- $\rho$ -nitrophenol caproate	
<i>P. indicus</i>	0.005*	Spectrophotometric assay, $\alpha$ -para-nitrophenol glucopyranoside			Omondi and Stark, 1995
<i>P. monodon</i>			†	Triolein/agar emulsion clearing	Deering et al, 1996
<i>P. vannamei</i>	0.07	Spectrophotometric assay, $\rho$ -nitrophenol- $\alpha$ -D-glucopyranoside			Le Chevalier and Van Wormhoudt, 1998
<i>P. vannamei</i>	0.005*	Spectrophotometric assay, $\alpha$ -para-nitrophenol glucopyranoside			Omondi and Stark, 1995

\* Denotes figures that were estimated from values reported in graphs.

† Represents a positive reaction using a triolein/agar emulsion screening.

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## APPLICATION OF AN EGG PRODUCTION INDEX TO DETERMINE REPRODUCTIVE PERIOD OF THE BROWN SHRIMP *FARFANTEPENAEUS CALIFORNIENSIS* NEAR AGIABAMPO, SONORA-SINALOA, MEXICO

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**ABSTRACT** Using the percentage of mature females as an index, the reproductive period of the brown shrimp, *Farfantepenaeus californiensis*, off the coast of Sonora in Mexico, has been shown to occur throughout the year with two peaks of mass spawning. The objective of this study is to determine if a similar reproductive period occurred off Agiabampo, at the Sonora-Sinaloa border. By combining 3 indices of mature females: size structure, abundance and fecundity at size, a single egg production index (EPI) was calculated. Monthly samples from January through December 2002 were collected during 5 continuous days around the full moon (full moon  $\pm$  2 days). Trawl net tows were used at depths from 1–9 m, 2 km north and south of the mouth of a coastal lagoon. Using the EPI, a single reproductive peak, May to August, was found, with May as the most important egg production month. On the other hand, the percentage of mature females showed a two peak reproductive period, one more intensive from May to August and the second one less intensive in November. We concluded that the best determination of the reproductive period of the brown shrimp required the use of three indices. Particularly off Agiabampo, this species has a well-defined reproductive period with just one massive spawning peak.

**KEY WORDS:** reproductive cycle, egg production index, shrimp, *Farfantepenaeus californiensis*, Gulf of California, Agiabampo

### INTRODUCTION

Most studies about reproductive patterns in shrimp are based on the percentage of mature females (Mathews 1981, López-Martínez et al. 1999, López-Martínez et al. 2005, Leal-Gaxiola et al. 2001). This is a biased index of population reproduction and must be combined with an index of mature female abundance and fecundity-at-size data (García 1985). If one wants to find the most important period for population renewal, the studies of the reproductive biology of shrimp must take into account more than just reproductive percentage of mature females. Among others indices that have been considered for this target, we found a gonadal index, egg production for the population (Oh & Hartnoll 1999).

Reproductive studies of brown shrimp *Farfantepenaeus californiensis* (Holmes, 1900) are few (Olguín-Palacios 1968, Edwards 1978, Mathews 1981, Barreiro-Güemez 1986, Méndez-Tenorio 1986, Garduño-Argueta & Calderón-Pérez 1994, Leal-Gaxiola et al. 2001, Aragón Noriega & Alcántara Razo 2005). Using only the percentage of mature females, the reproductive period of brown shrimp was determined as continuous throughout the year with two peaks of massive spawning. In these reproductive studies, 2 main sources of data have been used: daily packing-plant sampling from commercial catches (September to May) off Sonora, (Leal-Gaxiola et al. 2001) and data from research cruises that evaluated shrimp populations off Sinaloa (Barreiro-Güemez 1986, Garduño-Argueta & Calderón-Pérez 1994). Sinaloa and Sonora comprise the eastern coast of the Gulf of California (Fig. 1).

The daily packing-plant data did not include all months of the year, but comprised a long data series (Leal-Gaxiola et al. 2001),

and for this reason many reproductive studies were based on these data. A disadvantage of this data is that abundance could not be evaluated but only percentage of mature females. On board sampling commercial trawl vessel are scarce, and temporally sporadic (Aragón Noriega & Alcántara Razo 2005). The advantage of on-board sampling is that an evaluation of abundance of mature female shrimp can be made.

Fecundity of brown shrimp has been described with a power equation with a value around 3 ( $F = 0.0962 L^{2.9642}$ , Mathews 1981). The quantity of eggs spawned by ripe females increased because of the increasing size of mature females (Mathews 1981).

Although the fecundity and reproductive pattern of brown shrimp off Sinaloa and Sonora have been determined, fecundity-at-size, abundance and size structure of mature females have not been combined in previous studies. The application of egg production index (EPI) in penaeid shrimp was proposed by Courtney & Masel (1997). They found that this index is a more quantitative measure of egg production in a population at a particular sampling station or sampling time. Aragón-Noriega (2005) used an EPI calculated from size structure, fecundity-at-size, and abundance of mature females to determine the maturity period of blue shrimp *Litopenaeus stylirostris* (Stimpson, 1874) in central Gulf of California.

Studies on reproduction of exploited species are critical not only for fisheries managers, but they also have a general scientific interest because spawning is the basis of population renewal and conservation of the stocks. With this in mind, we should look for more precise methods that can help to determine the maturity period of species with economical or ecological importance.

The objective of this study is to determine the reproductive period of brown shrimp off Agiabampo, at the Sonora-Sinaloa

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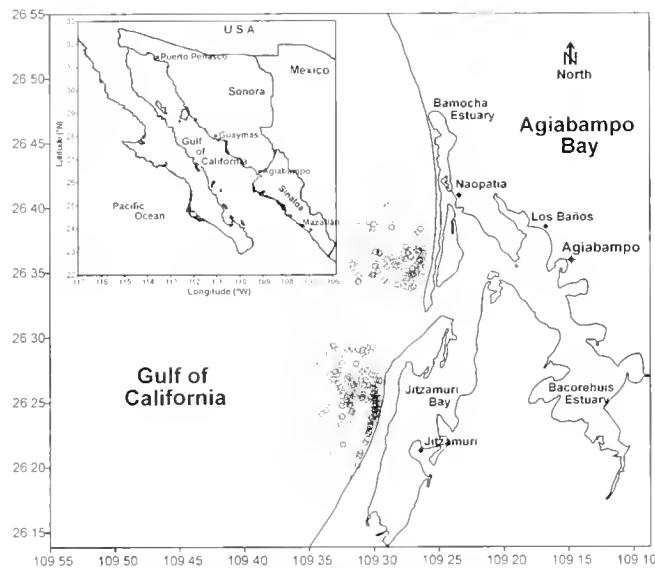


Figure 1. Study area and sampling zone.

state border, by combining the fecundity-at-size, abundance and size structure of mature females indices into a single index, EPI.

## MATERIAL AND METHODS

### Study Area

The Estero de Agiabampo (26.3°N, 109.3°W, Fig. 1) is a relatively long and narrow barrier lagoon created by sediment deposition of longshore currents. The broad region has a subtropical desert (BWh) climate (García 1988) and the sea surface temperature averages 30.4°C in July and 19.5°C in January.

### Sampling

From January to December 2002, shrimp samples were taken each month for five continuous days (full moon  $\pm$  2 days). Collection was done with small boats, about 7 m long. We used trawl nets with a 15-m mouth and 32-mm mesh. Tows were made every 1.5 h at flood and ebb tides. This was done at depths from 1–9 m at locations ranging up to 2 km north and south of the mouth of the coastal lagoon (Fig. 1).

Sex, gonad maturity, total length (tip of the rostrum to tip of the telson)  $\pm$  1 mm and abdominal length of the samples was determined. Degree of gonad maturity was determined with the morphochromatic scale, comprising four stages (Leal-Gaxiola et al. 2001). For this study the specimens were grouped into immature (Stages I and II) and mature (Stages III and IV) categories.

Abundance of mature females was determined as specimens per hectare. This was done using a sweeping area described in Sparre & Venema (1995). Satellite geo-positioning devices locate the beginning and ending position of each trawl. The total of mature females was divided by the calculated area (in hectares). Fecundity-at-size was determined by the equations mentioned in Mathews (1981).

Egg production index (EPI), similar to one proposed by Aragón-Noriega (2005) was determined by combining size structure, fecundity-at-size and abundance of mature females. The EPI is formed as follows:

$$EPI = \sum_{i=1}^n F(PL_{ci}a)$$

where: EPI is the egg production index,  $i$  is the  $i$ th size class,  $F$  is fecundity at size,  $L_{ci}$  is the mean value of length of class  $i$ ,  $P$  is proportion of class  $i$  in a particular month, and  $a$  is the abundance of mature female in particular month.

## RESULTS

A total of 5,780 specimens of all stages were collected (3,723 females and 2,057 males), yielding a female:male ratio of close to 2:1. However, for mature specimens, the ratio was 3:4, female:male ratio.

### Period of Sexual Maturity

The percentage of mature males was more than 50% during the complete sampling period (Fig. 2). We found one period (May to August) in which the percentage of mature females was near or greater than 40% (Fig. 2). The other months in which mature females appeared were November (17%), April (10%) and February (1%).

### Abundance

Monthly abundance of mature females determined by the sweeping area method were as follows: April, 29 females  $ha^{-1}$ ; May, 176 females  $ha^{-1}$ ; June, 97 females  $ha^{-1}$ ; July, 82 females  $ha^{-1}$ ; August, 58 females  $ha^{-1}$  and November, 5 females  $ha^{-1}$  (Fig. 3). These results show that spring and summer is the most important reproductive period for brown shrimp, using abundance and percentage of mature females.

### Frequency of Sizes

Total length of mature males ranged from 90–160 mm, averaging  $123 \pm 1$  mm and a 10% coefficient of variation. Total length of mature females for the entire study period was 110–190 mm, averaging  $151 \pm 1$  mm with an 8% coefficient of variation. Male and female sizes were significantly different ( $F_{(1,3100)} = 3255$ ,  $P < 0.05$ ). Frequency of total length groups for May to August is

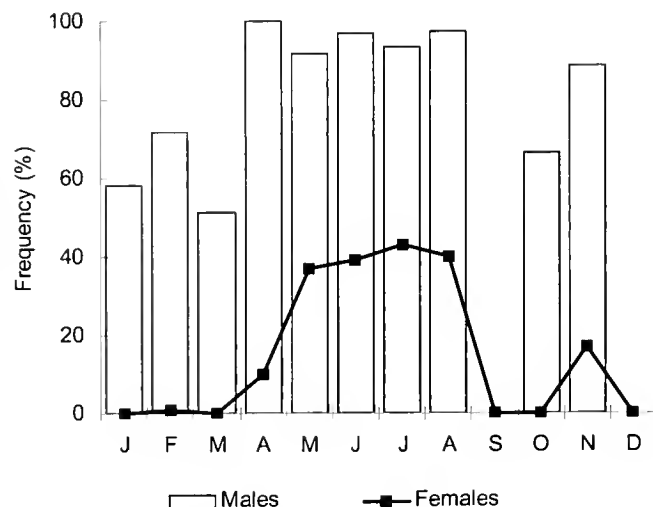


Figure 2. Monthly frequency of mature females (Stages III and IV) and males (Stage II) of the brown shrimp *Farfantepenaeus californiensis* off Agiabampo, Sonora-Sinaloa.

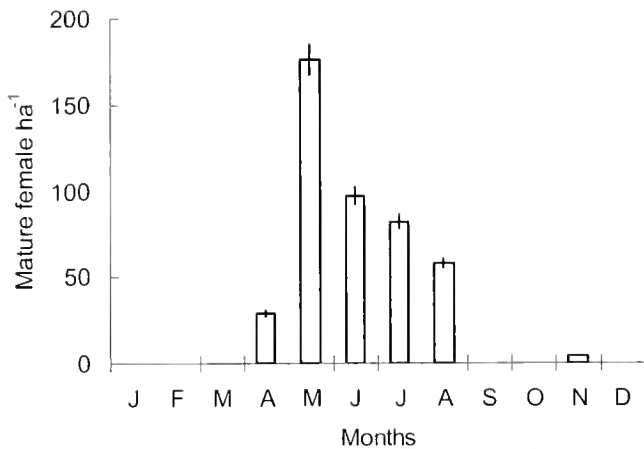


Figure 3. Abundance of mature brown shrimp females *Farfantepenaeus californiensis* off Agiabampo, Mexico.

shown in Figure 4. The modal size group was 44% greater than 165 mm in May. For June, July and August the percentage of specimens in the modal size group was 24%, 12% and 15%, respectively. Average total length of mature females was significantly greater in May ( $F_{(3, 1428)} = 47.96, P < 0.05$ ). No significant differences were found in June, July and August (Fig. 5).

#### Eggs Production

The EPI in eggs $\cdot$ ha<sup>-1</sup> did not match with months of higher percentage of mature females. EPI indicates that May is the most

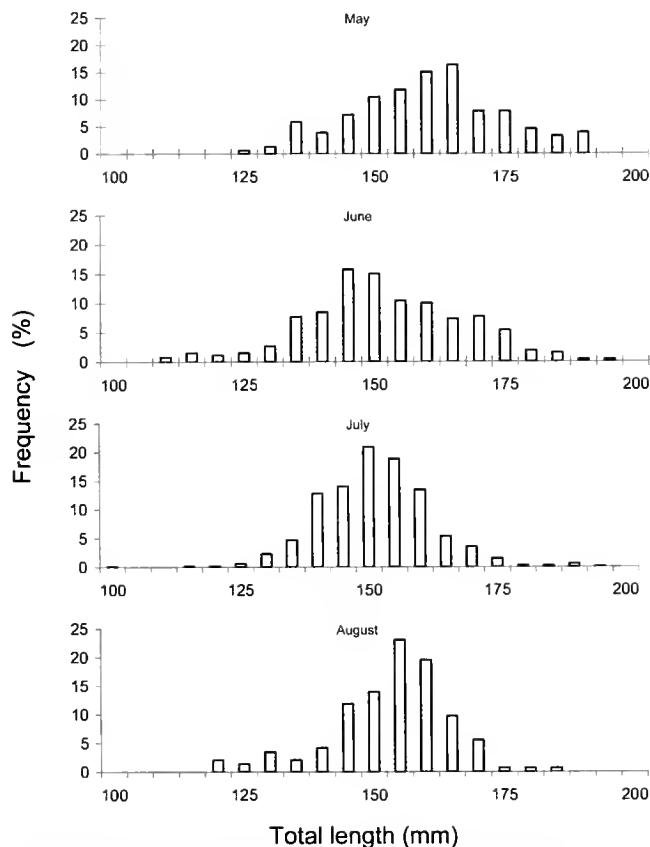


Figure 4. Size-structure of mature females (Stages III and IV) of brown shrimp *Farfantepenaeus californiensis* from May to August.

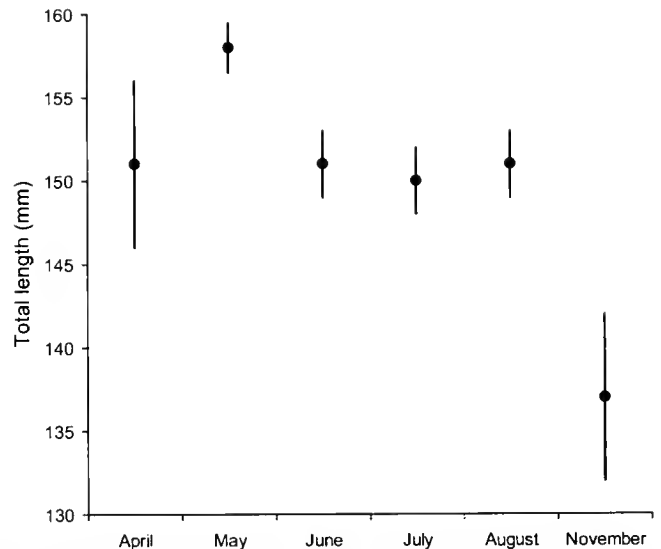


Figure 5. Comparison of average size between mature females (Stages III and IV) of brown shrimp *Farfantepenaeus californiensis* off Agiabampo, Sonora-Sinaloa

important month of potential egg production, followed by a clear negative tendency of EPI (Fig. 6). In May more than 60 million eggs $\cdot$ ha<sup>-1</sup> was the potential production on average. From June to August the average of potential eggs $\cdot$ ha<sup>-1</sup> were not more than 30 million. These results highlight the importance of using more than a single index to determine reproductive period for brown shrimp.

#### DISCUSSION

The reproductive period of brown shrimp *F. californiensis* off the coast of the Estero de Agiabampo is well defined as late spring to early summer, as quantified by using EPI. Leal-Gaxiola et al. (2001) found the reproductive period off southern Sonora as continuous during the year with two peaks of massive reproduction. However, those authors did not include data from June to September. For this reason their results are not adequate to determine the reproductive season for brown shrimp in that zone. The study of Leal-Gaxiola et al. (2001) could be easily combined with those of Méndez-Tenorio (1986) because this author sampled from June to January in the same zone and in the same years. Data from that study show an increasing percentage of mature females from June to August and decreasing percentage of mature females from August to January. The combination of data from the two studies

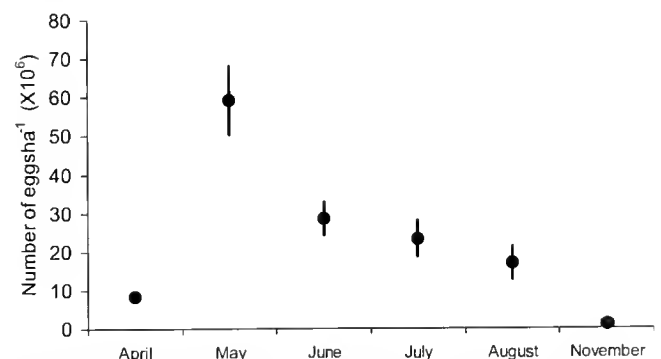


Figure 6. Eggs production per hectare of brown shrimp off Agiabampo, Sonora-Sinaloa.

yield a result similar to our findings. Leal-Gaxiola et al. (2001) found the times when the percentage of mature females was increasing and decreasing, but they erroneously concluded there were two periods of mass reproduction.

We found a single period of high rates of reproduction by using the percentage of mature females, similar to results found off Guaymas by Mathews (1981). He found mature females from February to November but a higher percentage from May to August. The reproduction period of brown shrimp reported off Mazatlán, Sinaloa is either continuous, with two periods of higher intensity (Garduño-Argueta & Calderón-Pérez 1994) or bimodal with a greater peak reproductive activity from March to May and a lower peak in October and November (Barreiro-Güemez 1986). García (1985) reviewed various types of spawning seasonal patterns of many shrimp species from around the world. He concluded that in spite of the shortcomings of the method (percentage of mature female) used for quantifying spawning activity, it seems that a bimodal pattern is most frequent. The main shortcoming of the percentage of mature female method is that overall population abundance of mature female is not taken into account but only a relative percentage of an unknown number per area (e.g., km<sup>-2</sup>, ha or other measurement).

García (1985) also concluded that reproductive behavior of shrimp species respond to transition periods of temperature. In the Gulf of California well-defined periods of transition in temperature occurs from spring to summer and from fall to winter (Aragón-Noriega & Alcántara-Razo 2005). So, conclusion that brown shrimp display a double peaked spawning pattern where accepted mentioned that the reproductive period (determined by the percentage of mature brown shrimp females) in the Gulf of California depends on the SST.

Because the percentage of mature females is a biased index of population reproduction (García 1985), we combined 3 indices: abundance, fecundity and size structure of the mature female brown shrimp, representing the first published index for this species. The only other study of EPI application was conducted on *L. stylirostris* (Aragón-Noriega 2005). In May we found 44% of the mature females over 161 mm TL. From the fecundity-at-size pro-

posed by Barreiro-Güemez (1986), 44% of the females could be spawning 800,000 or more eggs. Though the higher percentage of mature females was collected in July, we propose that May is the most important month for the reproductive period of brown shrimp off Agiabampo, because the highest EPI was found this month.

The percentage of mature females in November suggests a second, less intensive spawning period, but EPI showed that only a 1-million eggs•ha<sup>-1</sup> was the potential production, on average, in that month.

To determine the actual maturity period of any species we should take into account all shortcomings of the available methods. A problem for an exploited species is the specimens that remain unaccounted for after the fishery season. For the case of the brown shrimp near Agiabampo we surmise that if the reproductive behavior of this species responds to transition periods of temperature (García 1985) the abundance of mature female remaining after the trawl fishery is too low to be considered as a two-peaked pattern species. The use of EPI could help to redefine the actual maturity period for those two groups of penaeid shrimp, even if the second period of spawning for browns has been greatly diminished by fisheries activities.

This study clearly shows that results from one area cannot be extrapolated to other areas of the Gulf of California. Also, we found that current commercial catch data are not adequate for determining the reproductive pattern of brown shrimp. We agree with the proposal of García (1985) to use at least two other indices, and not just the percentage of the mature females in samples. We conclude that the appropriate combination of indices include size structure of mature females, abundance of mature females and fecundity-at-size to determine the reproductive period of brown shrimp. Specifically, those three indices for the brown shrimp off Agiabampo show a single maturation period from May to August.

#### ACKNOWLEDGMENTS

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## COMPARISON OF DOMOIC ACID CONCENTRATION IN KING SCALLOPS, *PECTEN MAXIMUS* FROM SEABED AND SUSPENDED CULTURE SYSTEMS

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**ABSTRACT** Domoic acid (DA) the toxin responsible for amnesic shellfish poisoning (ASP) has proven problematic for king scallop *Pecten maximus* fisheries and aquaculture in Ireland. Toxin concentration in hepatopancreas of individual scallops and composite samples of gonad and adductor muscle of scallops suspended from a submerged longline, 2 m beneath the water surface and on a seabed site, 12–15 m beneath the suspended scallops were monitored from February 2003 to February 2004 at an aquaculture site in Clew Bay, Ireland. DA concentration in hepatopancreas of scallops from the seabed and longline peaked in April 2003, individual concentrations reaching 1037.1  $\mu\text{g}\cdot\text{g}^{-1}$  and 1212.6  $\mu\text{g}\cdot\text{g}^{-1}$  respectively. No statistically significant differences were exhibited between DA concentration in hepatopancreas of seabed and longline scallops except on 1 of the 10 sampling occasions. Slow depuration of DA toxin from hepatopancreas occurred from April 2003 to June 2003 and the concentration remained relatively stable from June 2003 to Feb 2004. Interpretation of DA concentrations in gonad were complicated by the lower concentrations recorded and the variable size of the gonad caused by the reproductive cycle over the 12-month study duration. DA concentrations in adductor muscle were below the limit of detection throughout the investigation. In summary DA concentration between scallops held in suspension or maintained on the seabed exhibited minor difference, and routine monitoring samples could be collected annually and held in suspended culture systems rather than using more expensive diver collection for sample procurement.

**KEY WORDS:** amnesic shellfish poisoning (ASP), domoic acid, *Pecten maximus*, scallop

### INTRODUCTION

Amnesic shellfish poisoning (ASP) was first recorded in Canada in 1987 when over 100 people became ill after consuming mussels *Mytilus edulis* contaminated with the DA toxin. Early symptoms included nausea, gastroenteritis and vomiting followed within 48 h by neurological symptoms such as confusion, lethargy, disorientation and memory loss that persisted indefinitely; three of those affected in this outbreak died (Quilliam & Wright 1989, Todd 1993). The toxin, a small tricarboxylic amino acid belonging to the kainoid class of compounds, has been reported in 10 species of diatoms of the genus *Pseudo-nitzschia* (Bates 2000). Filter feeding bivalves such as scallops and mussels consuming these toxic phytoplankton species can accumulate the toxin to high concentrations (Zaman et al. 1997). Human consumption of shellfish contaminated with DA results in ASP.

Detection of DA in cultured mussels from Galicia, Spain in 1994 (Arévalo et al. 1997) and a range of Portuguese shellfish in 1995 (Vale & Sampayo 2001) led to the introduction of amendment 61/97 of EU Directive 91/492/EEC, which established the maximum allowable concentration of DA in whole shellfish or edible parts as 20  $\mu\text{g}\cdot\text{g}^{-1}$ . Extensive shellfishery closures have since been recorded in many European countries, perhaps the most significant being those applied to the king scallop fishery off the west coast of Scotland from 1999 onwards, which resulted in considerable financial hardship for scallop fishermen (Gallacher et al. 2001, Smith et al. 2005). Prolonged closures of scallop fisheries in Europe because of elevated DA concentration persisting in some instances for months or years (Arévalo et al. 1998, Fernandez et al. 2000), slow rates of depuration in this species, localization of the majority of toxin in the hepatopancreas and high inter-animal variation between scallops led to the introduction of EU Commission Decision 2002/226/EC. This allowed harvesting of scallops with a whole body DA concentration exceeding 20  $\mu\text{g}\cdot\text{g}^{-1}$  if the parts to

be marketed, principally the adductor muscle and gonad, contained less than 4.6  $\mu\text{g}\cdot\text{g}^{-1}$ . Scallop fishing from grounds that otherwise would have remained closed has been allowed, but this EU decision has necessitated that almost all the catch be processed in approved plants prior to sale. Restrictions on the sale of fresh scallops (in-shell), which have traditionally commanded a premium price compared with processed product, has resulted in a 2-tier market for fishermen, a high value one for fresh product, which can rarely be supplied and a lower value one for processed product.

In Ireland high DA concentrations in scallop recorded in late 1999 and early 2000 resulted in the closure of all scallop production/harvesting over the millennium period. Over the last 5 years, as in much of Europe, this toxin has proven particularly problematic in king scallop *Pecten maximus* fisheries. Recent DA concentrations of 618.2  $\mu\text{g}\cdot\text{g}^{-1}$  reported in April 2005 in mussels *Mytilus edulis* from the west coast of Ireland represented the first time the toxin had been detected above 50  $\mu\text{g}\cdot\text{g}^{-1}$  in this species. Interest in DA concentrations in scallop suspended in the water column compared with scallops on the seabed has been expressed from a number of perspectives. From an aquaculture perspective, scallops can be farmed either by sowing juveniles into seabed areas and harvesting at a later date or by more intensive culture in lantern nets suspended from submerged longlines. Both methods have been attempted in Ireland and in some bays both techniques have been used. Current legislation on the monitoring of DA refers to the number of shellfish to be used as a sample, the parts to be analyzed, the analytical technique to be used and the maximum concentrations. If differences in toxin concentration occurred between scallops produced by the two methods, then the possibility exists that scallops produced by one technique might be sold into a different market sector than the other, for example suspended scallops might have to be processed, whereas sown scallops from the seabed could be sold into the fresh market or *vice versa*. Given the higher value of fresh in-shell product, it is therefore conceivable that the production technique to be used in an area and the

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economics of scallop farming might be influenced by the likely toxin concentration in finished product. Such a possibility also raised the question that perhaps suspending scallops in shallower, phytoplankton-rich waters might increase rates of toxin depuration. Although bivalves that are actively feeding on nontoxic algae are likely to exhibit faster depuration (Bricelj & Shumway 1998), when mussels *Mytilus galloprovincialis*, contaminated with PSP toxin, okadaic acid, were transferred to a toxin-free zone the rate of depuration did not decrease significantly (Blanco et al. 1999). Nevertheless, the possibility that maintaining scallops in suspended culture nets until DA concentrations decreased to levels that allowed their sale into the more lucrative fresh market is worthy of further investigation for those faced with regular shellfish closures. The converse, whereby scallops suspended in waters containing a higher concentration of toxic species might accumulate more toxin than scallops on the seabed, should also be considered as a possible outcome of suspending scallops in the water column. Diver collection of farmed scallop has been proposed as a harvesting technique from intensive seabed plots. Though efficient as a means of scallop collection, such a technique is a slow method of harvesting, necessitating the holding of scallops for a period while a consignment is gathered. Concern has been expressed that during this holding period, scallops may accumulate toxins to levels such that their sale into the more valuable fresh market was no longer an option.

From a regulatory perspective, interest in DA concentrations in suspended versus seabed scallops centered on sample collection. In some inshore aquaculture sites in Ireland, divers are used for the collection of scallops for routine toxin monitoring. Recently introduced safety regulations for professional divers have resulted in an escalation of the costs associated with sample procurement using such methods. In circumstances where no differences existed between seabed and suspended scallops, sample provision for routine monitoring could be performed much more economically using scallops maintained for the purpose in suspended culture nets.

In addition to DA production from a range of *Pseudo-nitzschia* species, a benthic diatom, *Nitzschia navis viridica* has recently been reported as a further source of the toxin (Kotaki et al. 2004). The distribution of this species is at present largely unknown. If in a comparative study of DA concentration in seabed and suspended scallops, higher toxin concentrations were recorded in seabed scallops, DA production by benthic species might be suspected as a possible contributory factor.

Given the significance of results regarding DA toxin concentration in seabed and suspended scallops to fishermen, aquaculture producers, regulators and scientists; this comparative study was undertaken over a 12-month period at an aquaculture site on the west coast of Ireland.

## MATERIALS AND METHODS

King scallop *Pecten maximus* were collected by diving from a seabed aquaculture site in Clew Bay, County Mayo off the west coast of Ireland and subdivided into 2 batches—a suspended batch, which was held in lantern nets 2 m below the water surface from a submerged longline and a seabed batch, which was returned to the seabed below the longline at a depth of approximately 12 m to 15 m (Fig. 1). Samples from both batches were provided for DA analysis at approximately monthly intervals from February 2003 to February 2004. Each sample comprised 12 individuals from each batch of mean shell length between 100 mm to 115 mm. On three

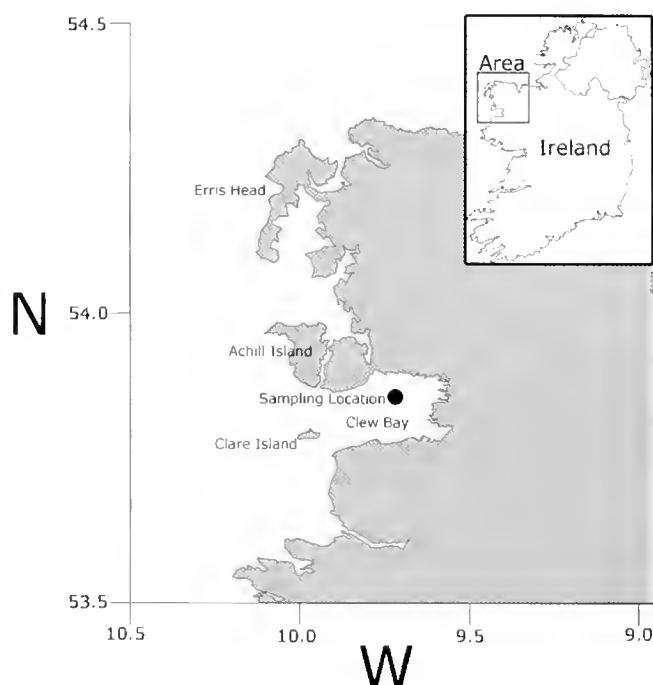


Figure 1. Sampling location in Clew Bay, County Mayo, Ireland.

occasions one or two shells in the longline sample were empty on arrival at the laboratory.

After shell measurement, scallops were dissected and the weight of total soft tissue and individual weight of hepatopancreas, gonad and adductor muscle recorded. The hepatopancreas of each scallop was analyzed individually to provide data on variability in DA concentration within each sample batch on each date. Composite samples of gonad and composite samples of adductor muscle from each sample group on each date were prepared for analysis, because previous studies suggested that DA concentration in these tissues would be much lower than in hepatopancreas; and hence differences, if present, between seabed and longline scallops would be more difficult to confirm because of the lower concentrations. Subsamples of composite tissues were analyzed in triplicate for DA toxin concentration.

DA was extracted from scallop tissue samples using procedures based on Quilliam et al. (1995) with several modifications. Extraction was performed from approximately 4.0 g of tissue homogenate with 16 mL of 50:50 extraction solvent (methanol:water) in a blender (Ultra Turrax T25, IKA-Works.) for 4 min at high speed. DA was extracted from each hepatopancreas individually. Where hepatopancreas weight exceeded 4.0 g, the organ was homogenized and a 4.0 g subsample used for extraction. After extraction, homogenates were centrifuged at 3,800 rpm for 30 min. A sample of supernatant was filtered using a methanol-compatible 0.45- $\mu$ m syringe filter and the combined concentration of DA and *epi*-DA in the filtered extract determined using a Shimadzu HPLC/UV and following equipment upgrade a Shimadzu HPLC/DAD (Mason Technology, Dublin). In those instances where the combined DA concentration exceeded that of the highest standard, the filtered extract was diluted with extraction solvent and the measurement of DA and *epi*-DA repeated. All solvents used were HPLC grade obtained from Lennox Laboratory Suppliers, Dublin. Mobile phase consisted of 10% acetonitrile and 1% TFA (trifluoroacetic acid) prepared with deionized water. HPLC flow rate was 0.5

mL.min<sup>-1</sup> and injection volume 20 µL. Calibration was performed externally using six DA calibration standards between 0.2 µg.mL<sup>-1</sup> to 10 µg.mL<sup>-1</sup> prepared from certified reference standard obtained from the NRC, Canada. Calibration curves were prepared for each sample batch and were always linear ( $R^2 > 0.999$ ).

Statistical analysis of all data was performed using SPSS, Version 12.

## RESULTS

Scallops used in this comparative study of DA toxin concentration were of a similar size (Table 1). Based on individual scallop measurements, the mean shell length and shell height for seabed scallops was  $108.8 \pm 3.9$  mm by  $96.4 \pm 3.9$  mm compared with  $111.6 \pm 5.2$  mm by  $99.4 \pm 4.6$  mm for longline scallops.

Mean DA concentration in the hepatopancreas of individual seabed scallops was  $326.4 \pm 169.9$  µg.g<sup>-1</sup> compared with  $313.9 \pm 214.6$  µg.g<sup>-1</sup> for longline scallops over the 12-month study at this location.

Mean DA concentrations in hepatopancreas and gonad of seabed and longline scallops on the 10 sampling occasions are provided in Table 1. DA toxin concentration in hepatopancreas of seabed and longline scallops exhibited similar trends over the 12-month duration (Fig. 2). The highest mean concentrations of DA in hepatopancreas were recorded on the second sampling occasion (April 3, 2003), individual DA concentrations reaching 1037.1 µg.g<sup>-1</sup> in seabed scallops and 1212.6 µg.g<sup>-1</sup> in longline scallops respectively. Comparison of the mean DA concentrations in hepatopancreas of seabed and longline scallops using a *t*-test demonstrated no statistically significant difference for 9 of the 10 samples. Only the sample from November 12, 2003 showed a significant difference ( $n = 23$ ,  $t = 3.263$ ,  $\text{sig.} = 0.004$ ) in the mean DA concentration between seabed scallops ( $298.3 \pm 89.4$  µg.g<sup>-1</sup>) and longline scallops ( $172.4 \pm 95.7$  µg.g<sup>-1</sup>).

DA concentrations in composite samples of gonad tissue never exceeded the regulatory limit of 20 µg.g<sup>-1</sup> and were considerably lower than concentrations recorded in hepatopancreas. Overall mean DA concentrations based on triplicate analysis of composite gonad samples were  $5.0 \pm 2.1$  µg.g<sup>-1</sup> in seabed scallops compared with  $3.8 \pm 1.9$  µg.g<sup>-1</sup> in longline scallops. DA toxin concentration

in gonads of seabed and longline scallops exhibited considerable fluctuations and dissimilar trends over the 12-mo study duration (Fig. 2). Because DA toxin concentration of gonads is influenced by both the mass of DA in the intestinal loop within the gonad and the mass of gonad tissue itself, the reproductive condition of scallops from both sample batches was determined in an attempt to understand the fluctuations and trends over the 12-mo study (Fig. 3). Relative gonad height (RGH) in both sample groups showed a similar trend with peak RGH at the end of May. Seabed scallops exhibited higher RGH than longline scallops, although differences between sample groups were not significant ( $n = 236$ ,  $t = 1.448$ ,  $\text{sig.} = 0.149$ ). The decline in RGH suggested a single prolonged spawning at this study location, although the use of monthly sampling for determination of the number of spawnings is not recommended (Slater 2005). Because of fluctuations in DA concentration within and between sample groups and varying reproductive condition over the 12-month study, the mass of DA in gonad was determined to examine if it better represented the changes in DA levels in gonad (Fig. 4). For comparative purposes the mass of DA in hepatopancreas was also determined (Fig. 4).

DA concentrations in composite samples of adductor muscle were below the limit of detection (LOD = 0.1 µg.g<sup>-1</sup>) in all samples over the 12-month study duration.

## DISCUSSION

DA concentrations in hepatopancreas were approximately 1 to 2 orders of magnitude greater than in gonad, hence if differences occurred between toxin concentration in longline and seabed scallops; such differences should be more easily recorded in those tissues having the higher DA concentration. Individual analysis of each hepatopancreas showed high inter-animal variability in DA concentration within each sample. Coefficients of variation (CV) of DA concentration in hepatopancreas of longline scallops ranged from 10.7% to 55.5% whereas that of seabed scallops ranged from 17.8% to 49.5%. The mean CV of DA concentration in hepatopancreas of longline and seabed scallops was 42.3% and 31.0% respectively. Similar high levels of inter-animal variability in DA concentration in king scallops have been reported (Campbell et al. 2001, Blanco et al. 2002, Bogan et al. 2006). Over the 12-month duration of the study similar DA concentrations in hepatopancreas

TABLE 1.  
Concentration of DA in hepatopancreas and gonad of seabed and longline scallops from Feb 03 to Feb 04.

Seabed					Longline			
	n	Shell Length (mm)	Conc. of DA in HP (µg.g <sup>-1</sup> )	Conc. of DA in gonad (µg.g <sup>-1</sup> )		Shell Length (mm)	Conc. of DA in HP (µg.g <sup>-1</sup> )	Conc. of DA in gonad (µg.g <sup>-1</sup> )
26-Feb-03	12	112.91 ± 2.71	272.58 ± 59.77	2.00 ± 0.24	12	111.58 ± 4.19	283.67 ± 62.69	2.25 ± 0.03
03-Apr-03	12	108.54 ± 4.08	697.96 ± 156.55	5.35 ± 0.64	10	112.50 ± 2.46	837.70 ± 250.84	7.10 ± 0.23
23-May-03	12	109.17 ± 3.69	457.68 ± 134.61	4.07 ± 0.46	10	107.80 ± 2.90	452.53 ± 202.18	7.12 ± 0.30
03-Jul-03	12	107.67 ± 2.57	216.50 ± 107.22	2.99 ± 0.43	11	108.70 ± 5.31	296.05 ± 114.60	2.24 ± 0.24
30-Jul-03	12	108.08 ± 3.34	332.70 ± 140.64	6.07 ± 1.21	12	113.83 ± 5.29	265.90 ± 56.09	1.96 ± 0.98
03-Sep-03	12	109.91 ± 2.84	304.98 ± 69.39	6.72 ± 0.21	12	109.75 ± 4.67	262.16 ± 111.62	2.58 ± 0.06
25-Sep-03	12	111.91 ± 2.42	223.68 ± 65.37	8.45 ± 1.01	12	113.42 ± 5.87	210.45 ± 99.96	3.54 ± 0.26
12-Nov-03	12	110.08 ± 1.62	298.33 ± 89.42	3.55 ± 1.12	12	114.64 ± 6.83	172.42 ± 95.67	3.09 ± 0.15
04-Dec-03	12	104.50 ± 2.97	238.98 ± 109.41	6.56 ± 2.65	12	111.08 ± 5.71	208.72 ± 46.59	4.36 ± 1.12
11-Feb-04	12	104.36 ± 2.98	245.24 ± 43.75	4.02 ± 0.66	12	112.92 ± 4.99	244.55 ± 103.88	3.76 ± 0.79

Values represent the mean ± standard deviation.

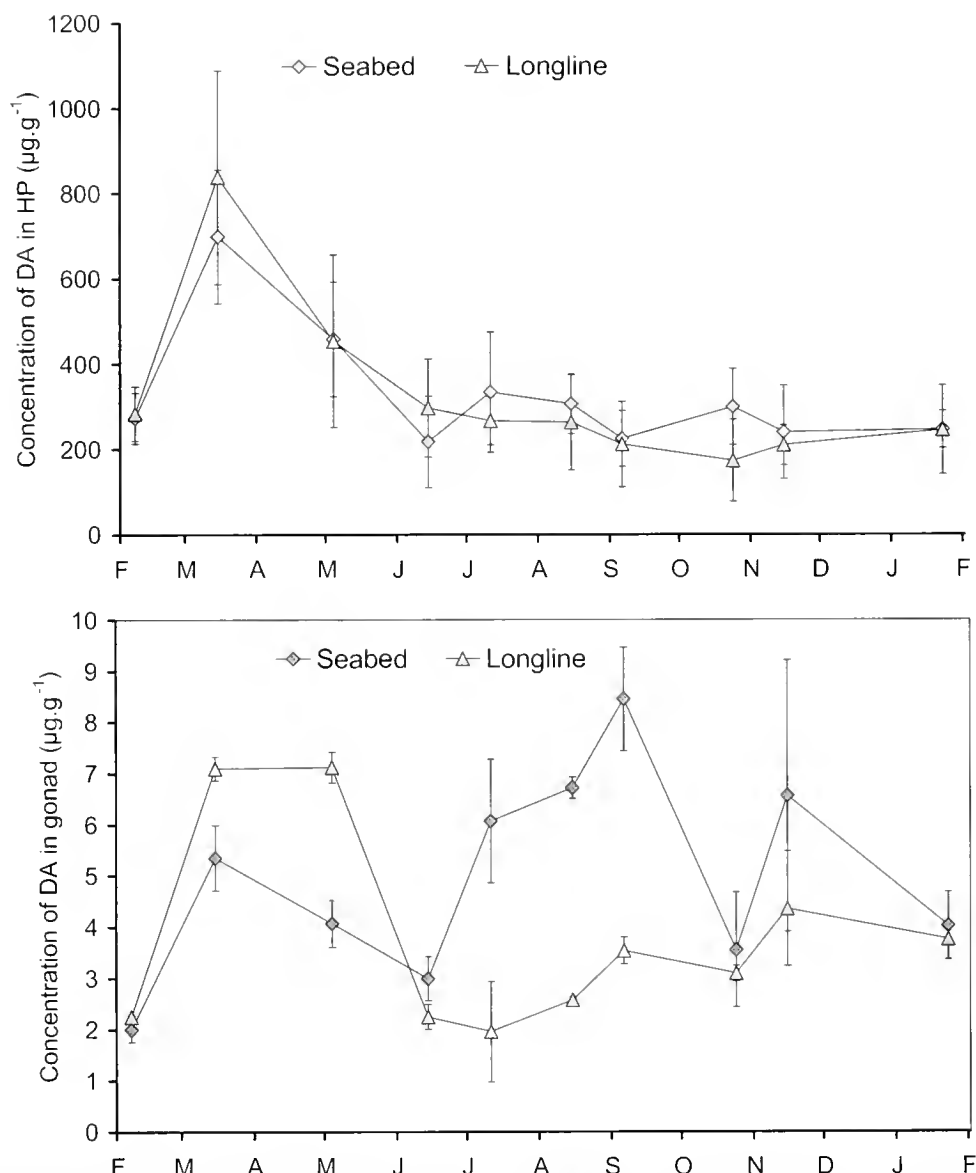


Figure 2. Concentration of DA in hepatopancreas (top) and gonad (bottom) of seabed and longline scallops from Feb 03 to Feb 04. (Each point represents Mean  $\pm$  St. Dev).

of longline and seabed scallops were reported and a statistical difference between the two sample batches occurred on only one occasion. Similarly no significant differences in PSP toxin concentration were reported in a comparative study between sea scallops suspended 2 m beneath the water surface and those on the seabed at 11 m depth (Haya et al. 2003).

Over the 12-month duration of the study both seabed and longline scallops demonstrated rapid accumulation of DA in hepatopancreas in March 2003, slow depuration during April 2003 to June 2003 followed by relative stability from June 2003 to February 2004. The fact that there was no statistical difference in DA content of hepatopancreas between longline and seabed scallops was somewhat unexpected. Reports of toxic *Pseudo-nitzschia* cells descending after a bloom from nutrient-poor surface waters into higher-nutrient mixed layers suggested that differential profiles in toxicity might have been anticipated between suspended and seabed scallops (Dortch et al. 1997, Trainer et al. 1998, 2000). A time

delay in PSP toxin accumulation in suspended and seabed scallops was suggested as toxic cells in surface waters descended towards the seabed as the toxic bloom subsided (Bricelj & Shumway 1998). In this study no temporal difference in peak DA concentration in hepatopancreas between longline and seabed scallops was recorded although this may have been caused by the long sampling intervals between February 26, 2003 to April 3, 2003 and April 3, 2003 to May 23, 2003.

Blooms of toxic *Pseudo-nitzschia* cells are often regarded as being widely dispersed throughout the water column, however they have also been reported as thin layers in the water column and following sinking as near-bottom thin layers, which may extend over large areas. Cell densities have been reported within these thin layers exceeding  $10^6$  toxic cells.l<sup>-1</sup> (Rines et al. 2002). The absence of a significant difference in DA concentration in hepatopancreas between longline scallops suspended 2 m beneath the water surface and seabed scallops from 12 m to 15 m depth, in all

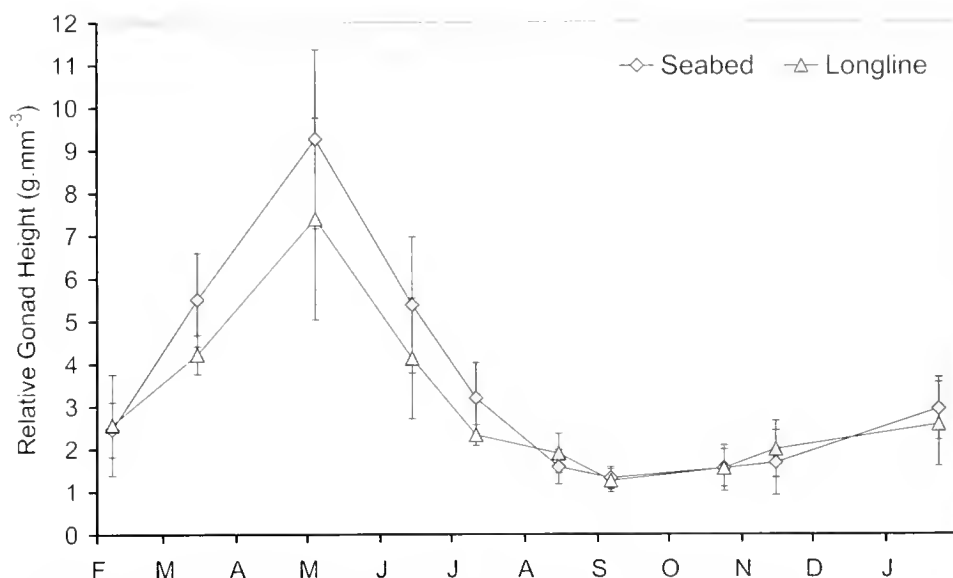


Figure 3. Relative gonad height of seabed and longline scallops from Feb 03 to Feb 04. (Each point represents Mean  $\pm$  St. Dev.).

but one of the samples, suggested that the source of DA toxicity was relatively evenly distributed throughout the water column in this location.

After the depuration phase during April 2003 to June 2003, concentrations of DA in the hepatopancreas over the remaining 8 month (June 2003 to February 2004) were relatively stable with minor fluctuations between  $170 \mu\text{g}\cdot\text{g}^{-1}$  to  $330 \mu\text{g}\cdot\text{g}^{-1}$  suggesting that no further depuration of toxin from hepatopancreas occurred, rates of uptake and depuration of DA were balanced or that the rate of depuration was very slow and not recorded over the 8-month period. Prolonged toxicity over an extended period rather than a continual decline in toxicity may have arisen from the continued development of small pulses of toxic *Pseudo-nitzschia* cells. Such pulses can result from *Pseudo-nitzschia* cells that had sunk after nutrient depletion being brought back into surface waters during upwelling events (Trainer et al. 2000). This replenishment of cells into surface waters has been proposed as a mechanism to explain why some blooms appear to persist for up to 3 mo in the field and might also explain why toxicity persisted throughout this study (Bates et al. 1989, Smith et al. 1990). Extended periods of toxicity attributed to very slow rates of depuration have also been reported in sea scallops, *Placopecten magellanicus* with PSP toxin over a 12-month period (Haya et al. 2003).

Many strains of domoic acid-producing diatoms have been reported, all belonging to the genus *Pseudo-nitzschia* except the benthic species *Amphora coffeaeformis* (Agardh) Kützinger (Bates 2000, Shimizu et al. 1989). Recently a benthic diatom, *Nitzschia navis-varingica*, collected from brackish water in Vietnamese shrimp ponds and identified as a new species has been demonstrated to be capable of DA production (Kotaki et al. 2000, 2004). Distribution of this benthic species is presently unknown although it has been reported in Japan and the Philippines and can be found in the water column (Lundholm & Moestrup 2000, Kotaki et al. 2004). The scallop toxicity results over a 12-month sampling period in this site provided no support for the hypothesis that DA production by benthic diatoms occurred.

DA concentrations in gonad were below the regulatory limit of  $20 \mu\text{g}\cdot\text{g}^{-1}$  throughout the study and never exceeded  $10 \mu\text{g}\cdot\text{g}^{-1}$  in

any composite sample of seabed or longline scallop gonad. Early results during the toxin accumulation phase in the hepatopancreas (March 2003) showed an expected increase in DA concentration in gonad. A corresponding decrease in DA concentration in gonad occurred from March 2003 to June 2003, although this was somewhat slower to commence in seabed scallops. During the period of relative stability in DA concentration in hepatopancreas (June 2003 to February 2004), DA concentrations in gonad fluctuated considerably and differences were exhibited between the two groups of scallops. To understand these fluctuations and between-group differences, the reproductive condition of the scallops using relative gonad height, a measure selected because it accounted for small differences in size between the scallop samples was examined. Because the DA is predominantly contained within the intestinal loop that passes through the gonad, it was expected that as the gonad increased in size DA concentration of gonad would decrease and *vice versa*. The results from the February 2003 to July 2003 period demonstrated the converse, as RGH increased and then declined after spawning, DA concentration in the gonad followed a similar trend. The results demonstrated that despite the variations in gonad size, DA concentration in the gonad was predominantly determined by DA concentration in the hepatopancreas, which exhibited a similar trend over the February 2003 to July 2003 period. The influence of gonad size on DA concentration in gonad was demonstrated by comparing toxin concentration between the scallop batches, higher RGH of seabed scallops being reflected in lower DA concentration whereas lower RGH of longline scallops was reflected in comparatively higher DA concentration. Fluctuations in DA concentration in gonad from Jul 03 to Feb 04 or variations between the two groups of scallop could not be explained by consideration of the RGH independently. In an attempt to explain the variations in DA concentration in gonad and gonad size, changes in the mass of DA in gonad were examined over the study duration. For comparative purposes changes in the mass of DA in hepatopancreas were also examined. Changes in the mass of DA in gonad over the 12-month period exhibited a similar trend to that for both the concentration and mass of DA in hepatopancreas. Because fluctuations in DA concentration of gonad from

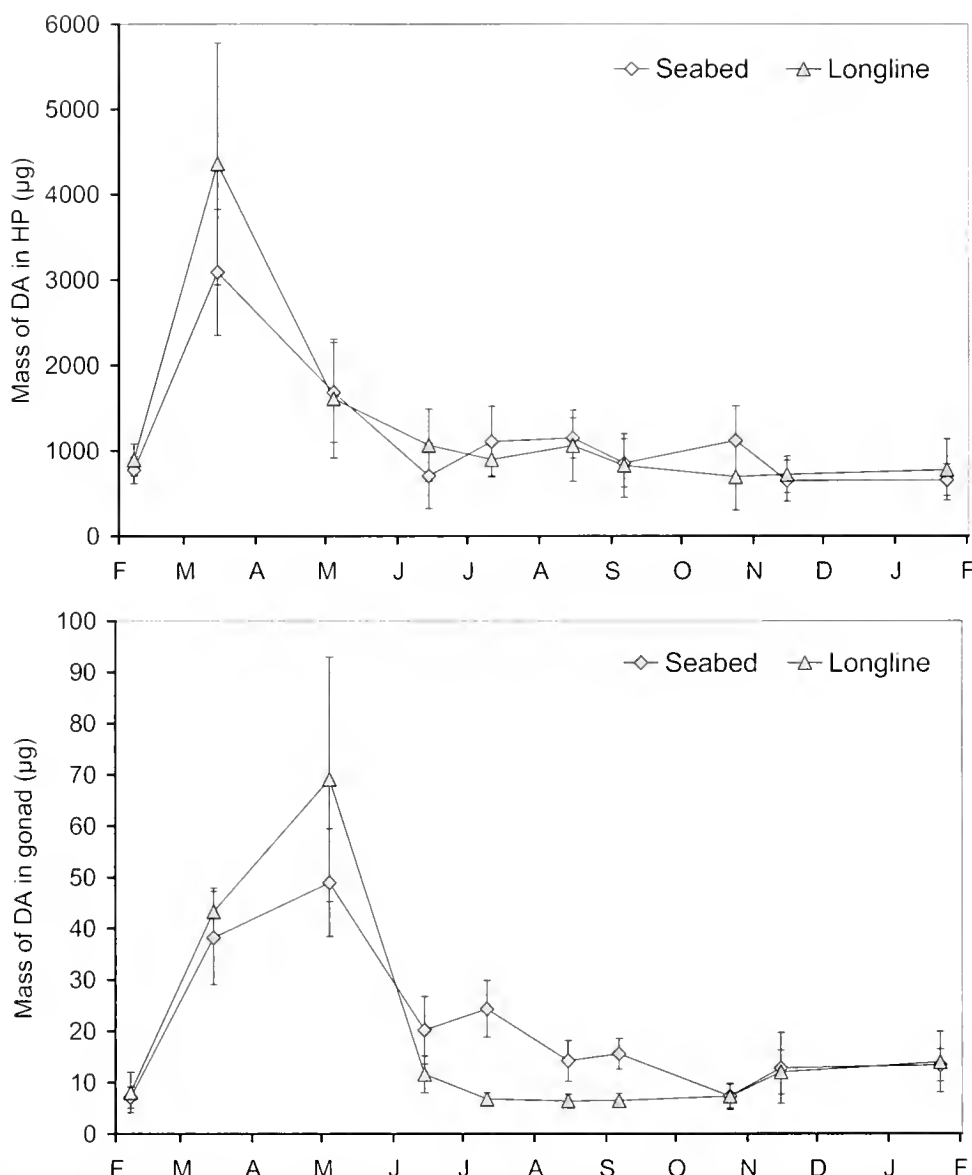


Figure 4. Mass of DA in hepatopancreas (top) and gonad (bottom) of seabed and longline scallops from Feb 03 to Feb 04. (Each point represents Mean  $\pm$  St. Dev.).

July 2003 to February 2004 could not be attributed to changes in gonad size, it was concluded that fluctuations during this period, and variations between the two scallop batches must have resulted from high inter-animal variability in DA concentration in gonad and experimental error. DA concentrations in gonad have exhibited high inter-animal variability in several other studies (Arévalo et al. 1998, Campbell et al. 2001). Similar difficulties to those encountered in this study in interpreting changes in DA concentration in gonad have been reported elsewhere (FSA 2001). Although regulatory monitoring of DA concentration in gonad for determining its suitability for entering the food chain is vital, the low concentrations compared with other tissues, variations in go-

nad size during the reproductive cycle and difficulties encountered interpreting results, suggest that its value in understanding temporal changes is questionable.

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## **SYMPOSIUM ON MOLLUSCAN FISHERIES AND AQUACULTURE**

*Papers presented at the:*

**World Congress of Malacology**

Perth, Western Australia

July 11–16, 2004



## SYMPOSIUM ON MOLLUSCAN FISHERIES AND AQUACULTURE

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The World Congress of Malacology was held on the campus of The University of Western Australia from July 11 to 16, 2004. The Congress, with a theme of *Molluscan Megadiversity: Sea Land and Freshwater*, attracted 300 mollusc workers from Australia and 41 overseas countries. Details of the congress can be found at: <http://www.mollusekey.com/perth>

The Congress provided an opportunity to develop a symposium on molluscan fisheries and aquaculture that highlighted current progress in the field, particularly in the Australasian region. A total

of 35 papers were presented: 27 lectures and 8 posters. Fourteen of the papers presented at the symposium are printed in full in this issue. A list of the other papers presented at the congress, and their abstracts, can be obtained from the listed website.

The symposium on molluscan fisheries and aquaculture and publication of the papers was generously supported by the Australian Fisheries Research and Development Corporation, the Western Australian Department of Fisheries Development and Better Interests Fund and the Journal of Shellfish Research. We acknowledge with appreciation the contribution made by the referees of the various papers and the considerable help from Sharon Brown of the WA Department of Fisheries in formatting the papers.

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Papers presented at the World Congress of Malacology Perth, Western Australia July 11 to 16, 2004



## AN ASSESSMENT OF THE ENVIRONMENTAL IMPACT OF WILD HARVEST PEARL AQUACULTURE (*PINCTADA MAXIMA*) IN WESTERN AUSTRALIA

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**ABSTRACT** Typical operating procedures used in the wild harvest pearl aquaculture (*Pinctada maxima*) industry in Western Australia are described as a basis for examining the potential environmental impact of the industry. A risk analysis workshop was held, which included industry representatives, marine scientists, regulatory agencies and conservation interests. The goal of the workshop was to document the main potential environmental and ecological risks that arise from the various activities carried out by the *P. maxima* industry. Thirteen environmental and ecological issues were identified across the *P. maxima* fishery. None were considered to be high risks; all were ranked as either moderate (23%) or low (77%). Moderate risk rankings included: introduction of disease from seeding; attraction of other fauna and introduction of exotic organisms. Low risks were: spread of disease; introduction of disease from hatchery; introduction of disease from translocation; impact to protected and endangered species resulting from entanglement; impact of habitat; impact to protected and endangered species resulting from farm lighting; nutrient impacts in sediment; perceived change in water quality; potential for litter and reduction of primary productivity. The low ratings given to disease risks took into account current strict regulatory controls for minimizing disease risks. The industry is considered to be environmentally benign. However, recommendations are made on how to further minimize risk.

**KEY WORDS:** pearling, aquaculture, environment, *Pinctada maxima*

### INTRODUCTION

The Western Australian pearling industry is a world leader, with a current average annual value of about \$125 million Australian. The Australian South Sea pearls produced are well regarded in the industry worldwide, and Western Australian companies have an enviable record for producing a high quality product. The pearling industry is unique in being a mix of wild capture and growout. It is the second largest successful aquaculture industry in Australia. In terms of dollar value, the fishery is second in Western Australia only to the western rock lobster, *Paralimnurus cygnus*. The industry is based on the pearl oyster *Pinctada maxima* (Jameson 1901), a bivalve mollusk species that has a widespread distribution in the Indo-Pacific and is not limited to Western Australia. Several other species of pearl oysters are cultured on a small scale in Western Australia, but are not covered in this article.

Pearl production has a long history in Western Australia, with the first recorded operations being in Shark Bay in 1850. In the early years, natural pearls were harvested from *Pinctada albina* (Lamarck 1819) collected intertidally or dredged from shallow water. Later shells became more valuable than the pearls and the industry was based on mother of pearl. No regulations were in place to sustainably manage the industry. As a result, pearl oysters were over collected, stocks became depleted and the fishery collapsed. The industry moved to the north coast of Western Australia where it centered on Broome and used the larger *P. maxima*. Shells were still the dominant product. By 1910 there were nearly 400 luggers and 3,500 people in the industry. However, when plastic buttons were introduced the industry crashed, and Western Australian pearling almost disappeared entirely during World War II. The industry made a comeback after the war with the beginning of the cultured pearl industry. The settlement of Kuri Bay started in 1956 and is still in operation (Shepherd 1975, Taylor 1985, Ander-

son 1996). In the early decades the fishery went through cycles of boom and bust, with the loss of men and boats through cyclones and other storms, loss of life and permanent injuries through diving mishaps and the bends, labor problems, racial tensions and fluctuating prices. Whereas there are still variations in the economic cycles, the industry is now highly organized and is geared to maintain sustainable production on an economically and environmentally sound basis. In this study we describe operations of the industry and assess its environmental impact.

### INFORMATION SOURCES

This study combines information gathered in two studies. For the first study (Enzer Marine Environmental Consulting 1998), an extensive literature search was undertaken to obtain information on the industry and the possible environmental effects of it. Copies of regulatory requirements were obtained from appropriate agencies. Discussions about the perceived environmental effects of the industry were held with governmental officers and other people within and outside of the industry. An 8-day field trip to the North West was undertaken to obtain first hand knowledge of the industry and its practices. The trip included an examination of a pearl oyster hatchery in Broome, 3 days observing fishing operations off Eighty Mile Beach, including several dives on various bottom types during the collection of pearl oysters and on shell holding areas to examine for any effects of the bottom longlines. One day was spent in Roebuck Bay at Broome on a bottom culture farm to observe the effects of shell culture and structures close to the bottom in various bottom types in the area. Three days were spent in King Sound to examine how surface longline farms operate, the cleaning process, methods of handling various wastes and rubbish, etc. Two types of farms were visited, a shore-based farm and two that are sea based.

For the second study (Jernakoff 2002), a site visit was conducted to evaluate current pearl industry practices and procedures. The objectives of the site visit were to: visit three pearl farms (Bynoe Harbour, Northern Territory; Kuri Bay, Western Australia

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and Talbot Bay, Western Australia), fishing vessels and interview staff to assess how the fishery operates and how closely its procedures and practices are in line with those required of an internationally recognized environmental management system; assess the degree to which existing practices and procedures are implemented; and report on the site visit and evaluate physical conditions, existing practices and procedures of a pearl industry in operation in the Broome, Darwin and Kimberley region, the area of Western Australia that is northeast of Broome. During the visit the observed farming activities included growout, seeding and harvesting. The physical conditions were observed in which the activities operated and the extent to which management systems were implemented.

An environmental and ecological risk assessment workshop was then conducted in September 2001 based on existing knowledge, considering all aspects of the fishery, identifying and prioritizing gaps in knowledge and producing a set of prioritized risks. The workshop included eight representatives of industry, five fishery scientists and managers, four staff from regulatory agencies and two community environmental representatives. The goal of the workshop was to document and risk rank the main potential environmental and ecological risks that arise from the various activities carried out by the *P. maxima* industry. This risk register is used to identify the underlying issues so that these may be addressed through the development of an appropriate management strategy to reduce the risk of deleteriously affecting the ecosystem in which the industry occurs. The aim was also to integrate the workshop with a broader ecological sustainable development (ESD) research program by providing a session of the workshop for the Department of Fisheries Western Australia (DFWA) to address environmental and ecological risks for the wild harvest component of the *P. maxima* fishery. The identification of key environmental issues assisted in determining if adequate information on those issues exists or if information is required after a review of national and international literature.

At the environmental risk assessment workshop, participants were asked to list all of the sources of environmental problems that could potentially be caused by the industry. The potential sources were then entered into a risk matrix of likelihood and consequence. Each of these was assessed against a score varying from 1–6. A likelihood of 1 was considered to be remote, whereas 6 was considered to be very likely. If the event did occur, the consequence was judged against a scale where 1 was negligible and 6 was catastrophic. Multiplying the two scores together provided their position on the matrix. Scores of 20 and above were assessed as being of high concern, 7–19 were moderate, and 6 and below were low.

#### BIOLOGY OF *PINCTADA MAXIMA*

The pearl oyster, *P. maxima* is widespread in the Indo-West Pacific. In Western Australia it has been recorded as far south as Shark Bay, but it is not commercially fished south of North West Cape. The species is a protandrous hermaphrodite, maturing first as males at an age of 3 to 4 years and a size of 110–120 mm. As they age, the animals undergo a sex change and become female. By 170 mm half of the animals are males and half are females. By 190 mm the population is entirely female. Because the animals can spawn every year, each individual functions as both a male and then a female for several spawning seasons. Very few animals are simultaneous hermaphrodites. The breeding season is long, from

September to October to April to May; most spawning occurs from the middle of October to December, with a smaller secondary spawning in February and March (Rose et al. 1990, Rose & Baker 1994, Knuckey 1995).

Gametes are spawned into the water column and fertilization is external. Females release from 2–12 million ova each, with a diameter of 60  $\mu\text{m}$ . Veliger shells form after 1 day, at an average size of 79  $\mu\text{m}$ . The planktonic veliger stage is a distributional phase that allows the young pearl oysters to colonize new areas if suitable bottom can be found. The pediveliger stage is reached by day 22 to 24. The majority of veligers start metamorphosing into juveniles on day 24. Settlement occurs over days 28 to 35. In nature, when veligers are ready to metamorphose they settle to the bottom and search for a suitable habitat. If an appropriate area is found, they settle on it and metamorphose into the juvenile stage. If no suitable settlement site is located within a short period the animals metamorphose and die (Rose & Baker 1994).

After settling to the bottom, *P. maxima* is byssate and attached to the bottom during its juvenile and adult phases of the life cycle. It requires a hard surface for the initial attachment, and once it is attached to the bottom the connection is permanent. *P. maxima* lives on shallow rocky pavements on the continental shelf where there are small crevices into which the young animals can settle and develop. Like most bivalves, pearl oysters are filter feeders. Growth rates are initially fast. Field measurements at Eighty Mile Beach have shown that the animals reach the minimum legal size of 120 mm shell height in their third year of life. They are fished for 3 to 4 years before growing to a size of 160 mm, beyond which they are no longer suitable for round pearl culture. Large oysters of 200 mm are 15–20 y old (Joll 1996). The animals can reach a maximum of 270 mm (Rose & Baker 1994).

#### MANAGEMENT OF THE INDUSTRY

Pearling and pearl farms for *P. maxima*, are managed by the Department of Fisheries Western Australian (DFWA) under the provisions of the Pearling Act 1990. The definition of *P. maxima* in the act includes any hybrids of *P. maxima* produced through laboratory technology. The Act is currently being rewritten. Other pearl oysters, such as *P. margaritifera* (Lamarck 1819), are regulated under the Fish Resources Management Act 1994. The industry is also regulated by a number of acts that deal with items such as marine safety, employment conditions, etc, which are not considered in this study. Under the Australian Commonwealth government's Environmental Protection and Biodiversity Conservation Act 1999, a fishery must be certified as environmentally sustainable by the Commonwealth Department of Environment and Heritage. This has been done for the maxima pearling industry. A copy of the assessment application and determination are available at <http://deh.gov.au/coasts/fisheries/wa/pearl/index.html>. Whereas culture and management methods are similar, the other species are not discussed in this study. The major fisheries regulations for *P. maxima* are described later.

Pearl oysters caught in the wild are seeded with a nucleus at sea in laboratory type conditions on board specially built vessels, then grown out in controlled sea lease areas for 2 years to produce cultured pearls. The wild stock of pearl oysters has thus been the basis of the industry. Several standard fisheries management procedures protect the stocks and ensure continuity of supply. Taking of pearl oysters for seeding is currently controlled by a quota system (total allowable catch is 572,000, but can be varied as

necessary) and additional measures, including size limits. Each operator has an annual quota of live pearl shell. In addition, the animals must be collected from specified zones within the fishery (Fig. 1). The minimum size limit for collection of pearl oysters is 120 mm, when the animals are 3 to 4 years old. There is no regulated maximum size, but in practice individuals are not taken over 160 mm because they are too slow growing to produce high quality pearls. This has a beneficial effect in helping to maintain the breeding stocks because the larger animals are females. Maximum sizes may be specified for particular areas of the fishery (e.g., Exmouth Gulf). Catch and effort data must be provided to DFWA on each dive made during the collecting season. Details are logged using square blocks of 10 nautical miles divided into subblocks of squares with sides of 2.5 nautical miles.

Pearl oysters are seeded after collection and held on the pearling grounds for a period of about 3 months before they are transferred to the farm leases. This allows the animals to recuperate after the stress of being collected, transported to the holding areas and having the nucleus inserted. After the nucleus is inserted, the shells are held in areas separated from those of other operators and areas being fished. Farms are also separated from each other, usually by 5 nautical miles as a counter disease method. However, if the licensee of an existing farm agrees, a new farm can be established as near as 2 nautical miles (FDWA 1997a). Additional reasons for having a clear separation between operators include providing each farm with opportunities for expansion and security of their equipment and pearl oysters.

In recent years hatchery-grown spat have been increasingly used to supplement wild stock. This provides a mechanism for

enlarging the industry in a controlled manner, ensuring a steady supply of stock and in the future potentially reducing costs of obtaining pearl oysters for seeding. Several hatcheries are now in operation. They are all subject to a stringent translocation protocol developed by DFWA in liaison with industry (FDWA 1997b). The hatchery technology provides increased potential for mixing genetically distinct populations during culturing. Johnson and Joll (1993) examined the genetic structure of *P. maxima* collected from 5 widely separated areas: Exmouth Gulf and Cape Bossut, Western Australia; Flat Top Bank and Oxley Island, Northern Territory and Thursday Island, Queensland. Most of the variation found was clinal between western and eastern populations, but comparisons between adjacent pairs of samples usually showed significant genetic differences. This includes the two locations in the Northern Territory that were only 320 km apart. Western Australian samples showed little subdivision over the 800 km from Exmouth Gulf to Cape Bossut. Johnson and Joll (1993) suggested that stocks are in general highly divided in northern Australia but that there are also substantial connections that occur in Western Australia over long distances. The 80,000 to 100,000 *P. maxima* transported annually from Western Australia to farms in the Northern Territory do not seem to have affected adjacent natural populations.

The Western Australian Department of Conservation and Land Management administers several types of marine reserves, and a formal assessment process is undertaken for any application for a pearling lease in a marine reserve. The decision on whether to grant lease approval in a marine reserve rests with the Minister for the Environment, not the Minister for Fisheries. The management areas are:

- Marine nature reserves are set aside for the protection of flora and fauna. Pearling activities are not permitted.
- Marine parks. A series of management zones is established in each marine park to determine the activities that can be undertaken in each zone. Pearling activities may be undertaken in general use zones and some special purpose zones but not in sanctuary and recreation zones.
- Marine management areas allow a broader management approach in the marine environment. Pearling activities are permitted.
- Land reserves include nature reserves, conservation parks and national parks. Land-based pearling activities are not considered to be compatible with the purposes and objectives of the land reserves and are not allowed.

#### METHODS USED BY THE FISHERY

##### Background

In 2004 there were 16 operators in the pearl industry in Western Australia, with locations from Exmouth Gulf to the Kimberley. Pearl oysters collected in Western Australia are also used to produce pearls at growout areas in the Northern Territory. Because of differing geography and size of the individual operations, there are differences in the approaches used to produce pearls, but all share common features. The techniques outlined later are generic for the industry, and may be modified by individual operators to adapt to local conditions.

During the late 1970s and early 1980s serious mortality of pearl oysters occurred in the transportation phase of the industry. The mortality was attributed to the bacterium *Vibrio harveyi*, which



Figure 1. Pearl Oyster (*Pinctada maxima*) Managed Fishery.

occurs naturally in the marine environment, including the water column, in sediments and in the guts of marine animals. The mortality occurred after transportation of the pearl oysters from collection areas to the lease sites. Instead of the usual 10% to 20% mortality, losses on lease sites were up to 80%. Surviving pearl oysters developed deformed nacre and were useless for half pearl or mother of pearl production. Poor water circulation and accumulations of mollusk feces on the bottoms of the tanks in which the oysters were transported were found to allow the number of bacteria to increase exponentially during transport. The possibility was also raised that circulation was not as effective on culture rafts as on long lines, and bacterial densities were higher. Whereas other infectious agents or causative factors could have been involved, it was concluded that pearl oysters were weakened during the low temperatures of winter, and they became infected when they came into contact with high bacterial concentrations (Wolf & Sprague 1978, Pass & Perkins 1985, Dybdahl & Pass 1985, Pass et al. 1987, 1988).

The mortality experience has made the pearling industry operators acutely aware that they are dealing with live animals that must be treated properly if high quality pearl production is to be achieved. A number of changes were made to improve treatment of the animals. The various processes are now staged to allow the animals to recover from each procedure (collection, transportation, seed implantation, etc.) before the next stage is attempted. Water circulation during transportation has been improved considerably, and the water in tanks is now exchanged about every 10 min. High-density raft culture has ceased and been replaced with long line techniques that use a lower stocking density.

The pearl industry makes use of a natural phenomenon that occurs in shelled mollusks. Shells produced by mollusks are a mixture of an organic matrix that provides the shape and structure and calcium carbonate that is the actual hard shell material. As a protective mechanism, many species secrete calcium carbonate around a foreign object that becomes lodged in their tissues. This seals the object off from the tissues, rendering it inert to the mollusk. The animal continues to secrete additional material onto the foreign object over time as it secretes additional shell. Many species of bivalves in a variety of groups, and indeed even some gastropods, produce pearls naturally. Because of their high quality, marine bivalves of the family Pteriidae produce most commercial pearls; some are also produced by freshwater species.

Because foreign objects, which lodge in tissues, vary considerably in shape and size; natural (keshi or seedless) pearls tend to be irregular in shape and lustre. Cultured pearls are produced by placing an inert foreign object into a pearl oyster so that it is completely surrounded by mantle tissue inserted in the gonad. The pearl oyster secretes shell material that seals the nucleus completely from the remainder of the body. Over time the animal continues to add layers to the pearl, continually enlarging it. The most common type of nucleus in use at present is made from the shells of freshwater bivalves from the Mississippi River of the United States, but there is an active search by the industry for an alternative source of nuclei.

Inserting a hollow plastic shape between the tissues of the animal and the shell of a pearl oyster in its final year of production makes half round or mabe pearls. The animal secretes shell over the foreign material, resulting in a half globe shape that is continuous with the remainder of the interior of the shell. Mabe pearls are large, with a thin covering of calcium carbonate over the base that had been inserted into the animal. All parts of the animal are

used after an individual oyster produces its final pearl: pearls are harvested, the shell is sold for mother of pearl, and the adductor muscle for edible meat.

#### *Obtaining Wild Caught Pearls*

Harvesting of wild pearl oysters occurs from Cape Leveque to Exmouth Gulf, with most collected off Eighty Mile Beach (Bowen 1991). Stocks of commercial quantities of animals at fishable depths (i.e., safe diving depths of <20 m) were surveyed between Broome and the Lacepede Islands in the 1980s (Penn & Dybdahl 1988). Other industry-based surveys have been made in other areas and are available to the industry but have not been formally published (Bowen 1991).

Sea bottoms on which pearl oysters are common are areas where there are crevices that allow the young animals to settle into a protected environment and a hard substratum for them to attach. The industry recognizes a variety of bottom types and has developed names for each. There are transitional zones between the bottom types. If a boat is traveling at a speed of 1 knot during a 1-hour dive, the total distance traversed will be about 1,800 m and there can be several minor changes in bottom type during the dive.

The bottom is typically a flat basement rock with very little relief. Fine sediment accumulates on it to a depth of a few millimeters, obscuring the underlying rock surface. A variety of organisms attach to the rock and provide the vertical relief up to 1 m off the bottom. There can be a substantial overlap in the fauna on the various bottom types; the type is determined by the dominant species present.

A low, round densely packed ascidian species, which lives attached on the bottom is the dominant species on potato bottom. In areas of heavy potato bottom the ascidians are almost completely dominant. Sponges are the next most dominant group, with a large variety of vase shaped, basket sponges and massive sponges up to 0.5 m high interspersed with smaller sponges of only a few centimeters. A variety of other attached fauna is also present, including sea fans, soft corals and sea whips. Despite the diversity of taxa present, total density is low. A very few corals (*Turbinaria*) are present. Faunal density rapidly decreases in areas where the rock is covered with sediment 2–3 cm deep. Bare sand patches can be interspersed between areas of potato bottom.

Garden bottom has a diverse faunal assemblage dominated by alcyonarian sea whips. Distance between the sea whips is variable, but on average they grow about 1 meter apart. The animals grow rapidly to up to 1 meter in height and quickly become encrusted with a variety of organisms, some very colorful, so the bottom does in fact resemble a garden. Sponges and other small encrusting organisms grow on the sea whips. Other than sea whips, a variety of sponges is present on the bottom. Ascidiarians are present, but are a larger species than that found on potato bottom. Other fauna present includes soft corals, sea pens and crinoids. No hard corals are present.

Whereas potato and garden bottoms dominate in the fishing area, several other bottom types are recognized by the industry, including collar, asparagus, etc. All share the common features of being located on a bottom with underlying rock that are inhabited by a wide variety of invertebrates. None of the habitats are in ecologically sensitive areas such as seagrasses, coral reefs or mangroves, which lack commercial quantities of pearl oysters.

The pearling vessel is a boat up to about 35 m long. Some of the modern vessels are custom made for the pearling industry,



others are modifications of existing boats. To save costs there has been a recent trend for the modern vessels owned by one company to be contracted to also collect pearl oysters for the smaller companies and all gain from economies of scale. The boat crew is usually from 10–12 people, including the skipper, engineer, deckhands, cook(s) and six divers. Booms are extended outward from each side of the vessel during the fishing for pearl oysters. On an average boat, three weighted ropes are hung vertically from each boom to a height of about 1 m off the bottom. There are thus six divers working at a time on the boat. A Morse code type of signal system is used by the chief diver to communicate with the crew on the boat, and control the boat speed, direction, height of the weights and divers, etc. On the rare occasion the weight strikes the bottom, the boat is signaled and the weight is raised. Not only does this practice prevent damage to the bottom, but if the weight is not raised it clouds the water, preventing the diver from fishing efficiently. A large bag near the weight is used to store pearls after they are collected. A good diver collects an average of 250 pearl oysters per day. The areas where pearls are collected are subject to extreme tidal ranges ( $\leq 9$  m), and consequently have very strong tidal currents. Diving is too difficult and dangerous during the spring tidal periods, and is only undertaken on the neap cycle when currents are substantially reduced and visibility is good. Fishing for live pearl oysters begins in January and continues for up to 5 months. The locations of patches have been logged and are revisited on a regular basis. The boat starts at one side of a patch and moves slowly with the tide across the patch at a rate of about 1 knot. Divers operate on hookah, or air supplied from a surface compressor; oxygen may be used at the end of the dive recompression to increase the rate at which nitrogen is purged from the body tissues of the diver. Each diver wears a smaller neck bag during the dive. As pearl oysters are collected they are kept in the neck bag until it is full. The pearl oysters are then transferred to the larger holding bag at the end of each weighted rope. The divers swim about 1.5 m off the bottom to obtain the maximum field of view. In murky water they swim closer to the bottom but are still off it.

At the end of each of the dives the pearl oysters that have been collected are recovered and measured. Under- and oversize oysters are returned immediately to the sea. As divers are paid only for correctly sized animals, novice divers rapidly learn to select sized animals. Shells are cleaned by simply scraping encrusting organisms off the pearl shell with a knife. A high-pressure hose is then used to wash the shells; no chemicals are used. The pearl oysters are placed in wire frame panels holding six animals each, and every panel is individually tagged to indicate which company has collected the shell and placed in a recirculating tank on board. When sufficient animals have been collected, they are taken to a holding area where they are carefully placed on the sea bottom in a marked area for later usage. The panels are attached at 900 mm intervals to lines, which may be several hundred meters long. Divers inspect the line on the bottom to ensure the pearl oysters are in the proper orientation. The bottom selected in this region is very similar to the fishing ground. Sponges are dominant, but there are also soft corals, sea fans and other fauna present, including some *Turbinaria* corals. The period of up to 2 months that the pearl oysters remain in the holding area minimizes the physiological effect of having been collected and allows the animals to recover before the nucleus is inserted. Additional holding areas are used because the boat works different areas.

### *Seeding the Pearl Oyster*

When they are to be seeded, the pearl oysters are recovered, and a piece of mantle tissue from another animal is inserted into the host oyster gonad along with the nucleus for the pearl. The operation is simple, but delicate. The inserted mantle tissue becomes part of the host oyster's tissues, creating a sac around the nucleus. If the oyster is subsequently used to produce a second pearl, the same sac of tissue is used (Joll 1996). After the operation the animals are returned to the ocean in panels at the holding area, again to minimize stress to the pearl oyster. After an initial recovery period of 7 to 8 days the pearl oyster panels are turned by divers over every 2 to 5 days. The turning helps to develop the sac around the nucleus and prevents the nucleus from breaking out of the tissue. The pearl oysters are x-rayed after 4 to 6 months to determine whether nucleus has been retained and the pearl has started to grow. If the nucleus has been rejected from the animal, the pearl oyster is operated on again in the following year or replaced with a hatchery shell (Scoones 1991, Joll 1996).

### *Transportation to the Farm*

Pearl oysters must be cleared from the holding areas by December 31 of every year. They are transported by boat to the pearl farm usually in the waters north of Broome. During transportation, the animals are maintained in seawater in holding tanks on the vessel with running seawater to keep the animals in as natural water conditions as possible. The water may be coarsely filtered to remove large particles. The rate of flow into the tanks changes the water about every 10 min. No feeding or chemicals are used in the transportation process. Each boat is capable of transporting 20,000 to 25,000 animals on a single trip.

### *Farming Methods*

The pearl oysters in the panels are removed from the holding tanks on the boat at the farm. The tanks are emptied and cleaned using a disinfectant before the boat returns to the holding area to obtain a second load of pearl oysters. After the animals have been transported to the pearl farm they are placed in panels on surface long lines into the ocean for a period of 2 years to allow the pearls to grow. When the pearls have been harvested, as many pearl oysters as possible are reseeded. Approximately 40% to 50% of the animals can be used a second time. Forty to fifty percent of these can be reused for a third time; some individual pearl oysters can be used for up to 8 years.

Two anchoring systems are used to keep pearl oysters in the water at the farm, based on the amount of exposure at the site. Most farms use surface longlines. This has the advantages of avoiding the use of divers, minimizing interactions with large salt-water crocodiles and allowing the use of less skilled workers for routine work. In the longline method, steel pegs are anchored up to 2 m deep in the mud of the sea floor. Longlines with surface buoys are used to provide flotation. Vertical lines with panels containing pearl oysters are hung from the buoys and are maintained well off the bottom to avoid fouling. The lines are at least 100 m offshore and are 20–30 m apart to avoid entangling adjacent lines if one breaks. An average line is 100 m long with panels every meter for a total of 600 pearl oysters on the line. This gives a density of only 0.2–0.3 pearl oysters per square meter. A less common method, the bottom farm, is used in areas of harder bottom in more exposed areas such as Roebuck Bay (Scoones 1991). On these farms gal-

vanized steel bars ("star pickets") are used to hold the panels. Surface floats are used only to mark the locations of the farm.

At the farm, the pearl oysters are removed from the water for several minutes only and mechanically cleaned every 4 to 5 weeks, and more frequently in the wet season when growth of fouling organisms is faster. Cleaning machines that use high-pressure water to remove as much growth as possible have been developed; calcareous encrustaceans may be mechanically scraped off; no chemicals are used in cleaning. Dead shells are removed during the cleaning operations.

#### Harvest of the Pearls

The temperature of the surrounding seawater has an important effect on the lustre and color of the pearl. These are best in winter, so the pearls are harvested during July and August (Scoones 1991). During harvest, suitable pearl oysters are reseeded with a new nucleus to begin the 2-year process of producing a new pearl.

#### ASSESSMENT OF ENVIRONMENTAL EFFECTS OF PEARLING

In total, 13 environmental and ecological issues were identified across the *P. maxima* fishery. No high risks were identified during the workshop; risks were ranked as either moderate (23%) or low (77%). Moderate risk rankings included: introduction of disease from seeding; attraction of other fauna and introduction of exotic organisms (Fig. 2). The low risks were: spread of disease; introduction of disease from hatchery; introduction of disease from translocation; impact to protected and endangered species resulting from entanglement; impact of habitat; impact to protected and endangered species attracted to farm lighting; nutrient impacts in sediment; perceived change in water quality; potential for litter (e.g., plastic zip tie tags, plastic bags, buoys) to enter water and reduction of primary productivity.

Introduction of disease from seeding was considered to be of moderate risk. Three related risks, introduction of disease from hatchery or from translocation and spread of disease, were con-

sidered to be low. The low ratings given to disease risks took into account current strict regulatory controls for minimizing disease risks. As discussed earlier, during the late 1970s and early 1980s serious losses occurred because of *V. harveyi* killing the oysters. The bacterium occurs naturally in the oysters, water and sediments. Crowded conditions and poor handling techniques used at the time placed the oysters under considerable stress, which allowed the bacteria to multiply and cause disease outbreaks. Once the causes were known, handling techniques were improved considerably and the pearl oysters were more widely spread in the growout areas. There is now emphasis in the industry on caring for the pearl oysters and the problem has not recurred. It should be noted that *V. harveyi* was not introduced by the pearling industry, rather the crowded conditions allowed it to spread. There are clear DFWA guidelines for procedures to minimize the possibility of introducing diseases through hatcheries. Also, equipment used in inserting the nucleus into the pearl oysters is sterilized before it is used in a different area.

#### Attraction of Other Fauna

Saltwater crocodiles might be attracted to the farms because of the lights and food wastes. However, these animals are dangerous, and activities that might attract them to the farms are minimized because of safety concerns. Small fish are attracted to the boats when pearl shells are being cleaned, and feed on portions of the material being removed from the shells. This is an intermittent activity, which occurs in a given spot only once every 3 to 5 wk, and then only in the daytime. The effect is transitory, and there is no longer-term buildup of fish populations.

#### Introduction of Exotic Organisms is a Major Concern in Australia

Once species become established in a new area they may be free of natural predators and other natural constraints on their populations. In these conditions a number of species have developed into plague proportions, which have caused severe environ-

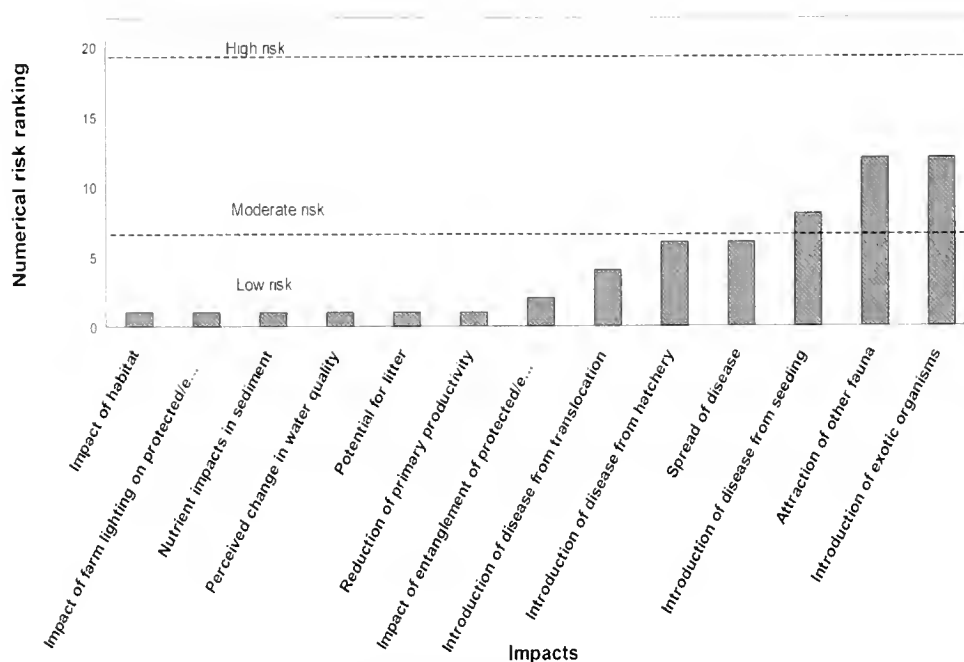


Figure 2. Risk ranking of impacts from pearl farm activities.

mental problems. Once a species becomes established, it is virtually impossible to be eradicated. The only reported successful eradication of a marine introduction worldwide occurred recently in Darwin Harbour. The mussel *Mytilopsis sallei* (Récluz 1849) was successfully removed from three small artificial boat marinas. The eradication was successful because the marinas use locks to allow vessels to enter and leave. They could be sealed off and chemicals introduced to kill the mussels (and virtually everything else) in the marinas (Willan et al. 2000). The issue of possible introduction of exotic organisms has two components, introductions by the pearling industry and introductions from other sources. The north coast of Western Australia is part of the tropical Indo-West Pacific biogeographical zone, which extends from the east coast of Africa across the tropical areas of the Indian and Pacific Oceans to Hawaii. Many species are widely distributed in the region, and some have ranges that extend over the full range. The north coast of Australia, including the full range of the *P. maxima* pearling industry, is part of the Indo-West Pacific (Wilson & Allen 1987). There are no major distributional barriers on the north coast of Western Australia; species tend to be distributed over the entire area if the proper habitat is available. Because the boats in the pearling industry operate within this zone, there is little likelihood of them introducing a species from overseas or eastern Australia.

#### *The Remaining Potential Effects of Pearling Were all Considered to be Low Risk*

Impact to protected and endangered species resulting from entanglement would arise if a protected or endangered species became trapped in one or more of the pearl lines. There are no known instances of this happening in the *P. maxima* industry, but there was a recent case in the black lip pearl (*P. margaritifera*) industry in the Houtman Abrolhos Islands on the west coast of Western Australia where a dead whale was washed ashore entangled in pearl lines. It could not be established whether the whale became entangled before or after death. Such occurrences do occasionally happen in the crayfish industry on the west coast, but there are tens of thousands of pot lines in the water during the 7.5 mo season. Entanglements in the pearl industry are at most very rare. Impact to protected and endangered species resulting from farm lighting is a related potential effect of the pearling industry. Oil rigs off the north coast of Western Australia are known to attract sea birds and turtles at night when their lights are on. However, the rigs have considerable lighting for safety and are worked 24 h a day. The small vessels and shore base farms of the pearling industry use only the lighting necessary for their activities, and are largely darkened for much of the night.

#### *Impact of Habitat*

Clearly establishing facilities for the pearling industry has some effect on the habitat. However the farms are small when compared with the vast distances on the north coast of Western Australia. They are also predominantly situated over mud bottom for maximum pearl growth. The major impact is the visual presence of the longlines, which are floating in the water, except for an anchor on each end, which is placed in the mud bottom.

Nutrient impacts in sediment, perceived change in water quality and reduction of primary productivity are three interrelated possible effects of pearl oysters being maintained on the farms for 2 years. To some extent they are contradictory. Possibly nutrient

impacts on sediment and perceived changes in water quality were considered to be a possible result of feces from the pearl oysters and residue from the cleaning process. Pearl oysters are sedentary animals with a low metabolic rate. At a density of 0.2–0.3 pearl oysters per square, there is unlikely to be much feces settling to the bottom. There is material cleaned off the shells every 3–5 wk. Again, the low stocking density of the pearl oysters suggests only a small amount of material is put into the system during cleaning. Portions are actively consumed by fish and the remainder is dispersed as it settles to the bottom. In any event, there is no net increase in nutrients, because the biological material was produced from nutrients already present in the water column. Reduction in primary productivity would occur because of the pearl oysters removing material from the water. Again, the effects would be negligible given the low stocking density of the pearl oysters and the dynamic environment of the north coast, where spring tidal ranges vary from 3–10 m, creating strong currents and considerable water flow.

#### *Potential for Litter (e.g., Plastic Zip Tie Tags, Plastic Bags, Buoys) to Enter Water*

The litter issue has 2 components: littering and loss of lines during storms. The industry has been very much aware of basic littering issues and has taken steps to minimize this through a code of practice. The northwestern coast of Australia is cyclone prone, with several storms occurring in an average year. If the storm passes near a pearl farm, lines and their attached buoys and panels are occasionally torn free. In such cases every possible effort is made to recover the panels because of their high value.

## DISCUSSION

The studies of Enzer Marine Environmental Consulting (1998) and Jernakoff (2002) examined the potential environmental effects of the pearling (*P. maxima*) industry in Western Australia but used very different techniques. However, both came to the same conclusion, that the environmental impacts of the *P. maxima* industry in Western Australia are small. This is accentuated when one compares the industry to the other two major aquaculture industries in Australia, sea cage farming of Atlantic salmon in Tasmania and southern bluefin tuna ranching in South Australia. Both of these industries involve feeding considerable amounts of food to active fish, creating potential problems with uneaten food and fish feces falling through the nets to the bottom in low tidal areas, where it is broken down by physical and biological processes. These problems do not occur in the *P. maxima* industry, where the pearl oysters filter feed naturally and are stocked at much lower densities in high tidal areas where there is considerable natural water flushing.

The Australian Commonwealth government passed the Environmental Protection and Biodiversity Conservation Act in 1999; the act was gazetted in 2000. Under this act the Commonwealth Minister for the environment must certify that a fishery is operated in an environmentally sustainable manner if the fishery is to export its product from Australia. There was a 4-year period during which the initial applications could be submitted and assessed. DFWA (2002) submitted an application based on Enzer Marine Environmental Consulting (1998) and Jernakoff (2002). The Commonwealth Minister assessed the fishery as environmentally sustain-

able on November 5, 2003. Certification is for a 5-year period, after which a fishery must be assessed again.

In Japan, Yokoyama (2002) provided a clear demonstration of the differential effects of fish and pearl aquaculture. Monthly samples of macrobenthos were collected from a fish farm and a pearl farm in Gokasho Bay from June 1995 to July 1996. The results demonstrated that the community structure of the two sites showed distinct differences with seasonal fluctuations. At the fish farm, azoic conditions were found from July to November. After December, diversity increased markedly through successive recruitments of small-sized species such as the polychaetes *Capitella* sp. and *Pseudopolydora paucibranchiata*, and the amphipods *Aeroides* spp. Macrofaunal density, biomass and species richness peaked from March to April. In contrast, at the pearl farm site, a higher diversity, including larger-sized species, and no clear seasonal fluctuations in abundance were found, and community structure was similar to that at the control site. These results show that there was a large impact on the macrofauna at the fish farm, but little effect on the benthic fauna as a result of pearl farming. Yokoyama (2002) suggested that the difference in the level of organic input between the two sites results in the differences in the dissolved oxygen content of the bottom water, sulfide content of the sediments and, subsequently, the macrobenthic assemblages. It should be noted that environmental effects of fish farming depend on site and management. Felsing and Glencross (2004) showed that macrofauna minimized organic accumulation on the seabed at one site in Western Australia.

There have been few other assessments of the environmental impacts of pearl aquaculture, possibly because of the lack of perceived impacts. The studies that have been undertaken are in agreement with the results of the present evaluation of the environmental effects of pearling in Western Australia. Cheney et al. (1995) examined farming of the Black-lip pearl oyster (*Pinctada margaritifera*) in the Tongareva Lagoon, Cook Islands, where commercial farming began in mid 1993 but found little effect. Abo and Toda (2001) examined the relationship between stocking density of the Japanese pearl oyster, *P. fucata martensii* (Dunker, 1850), and planktonic food density measured as the chlorophyll *a* concentration at Gokasho Bay, central Japan. Food density (as chlorophyll *a*) is determined by the balance among the phytoplankton growth, filtration of pearl oysters and outflow by water exchange. A model was developed, which simulated food density and growth of pearl oysters associated with change in the farming density. The calculated food density and growth rate of pearl oyster were higher when the farming density was lower, whereas the food density and the growth rate were lower when the farming density was higher. During the summer peak of chlorophyll *a*, the concentration was about 7 µg/L in areas of normal stocking density of pearl oysters, compared with about 8 µg/L where the stocking density was reduced by half. Increasing the density of pearl oysters to 7 times the *in situ* farming density decreased the phytoplankton density by three-quarters to about 2 µg/L. The authors concluded that filtration intensified by overcrowded pearl oysters decreases food availability, which then retards the growth rate of the pearl oysters. Their model also suggested that the present farming density of the pearl oysters is appropriate under the food condition of their habitat in Gokasho Bay.

Whereas there have been no reports of environmental damage caused by pearl farms, the opposite may be true: the presence of pearl farms may improve water quality. There have been a number of studies suggesting the use of edible oysters to remediate marine

water conditions. For example, Jones (1999) found that the Australian oyster *Saccostrea glomerata* (Gould 1850) acted as a biological filter in reducing phytoplankton, bacteria, total nitrogen, total phosphorus and total suspended solids by amounts ranging from 5% to 67%. Oyster excretion increased concentrations of dissolved ammonium, nitrate/nitrite and phosphate. However, these concentrations were in turn reduced by absorption by the macroalga *Gracilaria edulis*. Whereas the same benefits could theoretically accrue from the use of pearl oysters, in practice stocking densities used in northwestern Australia would be too low to have much effect. Qian et al. (1996) demonstrated that the growth rates of both *Pinctada martensi* and the red alga *Kappaphycus alvarezii* were higher in a coculture system than when the species were cultured independently. When temperatures were above 20°C the alga used nitrogenous wastes from the pearl oysters. Below that temperature algal growth was inhibited and there was no measurable effect. The authors speculated that the reason for improved growth rates of the pearl oysters was improved water quality.

Gifford et al. (2004) suggested that pearl farms could be used as biological filters in coastal areas with anthropogenic nutrient enrichment and pollution. Pearl oysters are capable of filtering water at rates of up to 25 l h<sup>-1</sup> g<sup>-1</sup> of dry weight tissue. They extrapolated published information to suggest that a pearl farm of 100 tons of pearl oysters could remove 300 kg of heavy metals and 24 kg of organic contaminants through deposition into the pearl oyster tissue and shell, and that up to 19 kg of nitrogen would be removed per ton of pearl oyster harvested. There would also be substantial removal of bacteria. Gifford et al. (2004) suggested the concentration of heavy metals and other pollutants in the tissues and shells would not be a problem because the material would be disposed of at land sites and would not be used for human consumption. Gifford et al. (in press) tested this hypothesis at a small *Pinctada imbricata* farm in Port Stephens, New South Wales, which produced 9.8 tonnes.yr<sup>-1</sup>. The farm removed 703 g of metals, 7452 g nitrogen, and 545 g phosphorus. The authors concluded that if production was scaled up to 499 tonnes.yr<sup>-1</sup>, extraction of nitrogen would equal that being produced by a small sewage treatment plant. Changing the harvest dates of the pearls to coincide with the period of peak condition of oyster tissues would substantially increase material removed from the water column. The suggestion is that pearls produced could be sold to provide funding for the remedial activities and a profit. However, there is no information available on the quality of the pearls that would be produced. The *P. maxima* industry in Western Australia is based on producing high quality pearls in natural conditions. It is doubtful that the high quality could be maintained in polluted areas. Further, the north coast of Western Australia, where the industry is located, has few towns and sources of anthropogenic pollution, so there is no possibility of using pearl oysters for environmental remediation in this area.

As indicated earlier, the possible introduction of marine pest species into Australia was assessed as having a moderate risk. Reasons are provided earlier in the study as to why this risk is considered to be low as a result of pearling activities. On the other hand, species that have been introduced by vessels not associated with the pearl industry may potentially affect the industry, and such introductions could have significant impacts. Whereas not all of the ports on the north coast of Western Australia have been surveyed, there are known to be a number of species that have been introduced. NIMPIS (2005) lists 43 introduced or cryptogenic marine species in Western Australia. Seven of these species are on the

north coast of the state, but the list is incomplete. For example, Jones (2004) records six barnacles as introduced to the Dampier Archipelago. Matsuyama (2003) presented an outstanding example damage, which can be caused by an introduced species. The dinoflagellate *Heterocapsa circularisquama* is a causative agent of red tide. It appeared in Japanese waters for the first time in 1988, then spread rapidly in the coastal waters of western Japan. By 2000 there had been 43 red tides caused by *H. circularisquama*, including 18 that damaged fisheries, including the manila clam *Ruditapes philippinarum* (Adams & Reeve 1850) Pacific oyster *Crassostrea gigas* (Thunberg 1793), pearl oyster *Pinctada fucata*, and the blue mussel *Mytilus galloprovincialis* Lamarck, 1819. Economic losses in shellfish aquaculture by direct killing of marketable products were estimated to be at least 10 billion-yen in the last decade; at current exchange rates this is approximately one hundred million US dollars. *Heterocapsa circularisquama* red tides primarily affect bivalve aquaculture. No harmful effects on wild or cultured fish, other marine vertebrates or any public health hazard have been recorded. This is a tropical species, which could potentially cause substantial effects to the pearl industry if introduced into Western Australia.

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## THE ROLES OF BACTERIA AND MICRO AND MACRO ALGAE IN ABALONE AQUACULTURE: A REVIEW

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**ABSTRACT** Abalone aquaculture is dependent on cultured algae to induce larval settlement and as a food source for the early life stages of abalone until formulated feed or macroalgae such as *Macrocystis* sp., *Porphyra* sp. and *Ulva* sp. are introduced into the growout system. In the natural environment, abalone larvae settle on coralline red algae, which provide one of the strongest and most consistent settlement cues available for abalone larvae. However, propagation of coralline red algae is not practical commercially. Abalone farms in Japan successfully settle abalone larvae (*Haliotis discus hannai*) on the green alga *Ulvellla lens*. *U. lens* also proved to be suitable to enhance settlement of cultured southern Australian abalone species (*Haliotis laevigata*, *H. rubra*). Most abalone farms in Australia are now growing *U. lens* for that purpose. *U. lens* is easy to culture, no specific facilities are needed and the alga can be grown on PVC settlement plates in commercial nursery tanks. However, *U. lens* has limited value as a feed for young postlarvae. Instead, cultured diatoms can be added after larvae successfully settle and start feeding. Juvenile abalone (>3 mm in shell length) can consume *U. lens* and grow rapidly on this alga. Diatom cultures and biofilms developing on settlement plates are not axenic and the role of bacteria in early postlarvae feeding is poorly understood. It has been suggested that bacteria may perform metabolic activities in the undeveloped gut of young postlarvae. At later stages of the nursery phase it becomes increasingly difficult to maintain adequate feed on the plates and this is still regarded as a significant bottleneck for the abalone aquaculture industry. Recent investigations have indicated that sporplings of macroalgae like *Ulva* sp. or diatoms that can provide more biomass may provide a suitable additional food source for juveniles (>3 mm in shell length).

**KEY WORDS:** abalone, abalone eggs, antibiotics, algae, bacteria, diatoms, growth, larval quality, lipids, settlement, *Ulva* sp., *Ulvellla lens*

### INTRODUCTION

Abalone fisheries (*Haliotis* spp.) produce high value, export-orientated products with about 50% of the world supply being provided by Australian fisheries in 1999 (Gordon & Cook 2001). The worldwide catch from abalone fisheries has declined by about 30% over a 10-year period from ca. 14,000 mt in 1989 to 10,000 mt in 1999, and consequently the interest in aquaculture products has increased substantially. The world production of abalone from aquaculture in 1999 was approximately 7,775 tonnes (Gordon & Cook 2001). Future production from the numerous farms and sites established, under construction or approved in several countries including Australia, could be even more substantial if the technology is improved.

In an aquaculture environment, abalone larvae are produced by spawning recently collected wild broodstock, or wild or farmed abalone broodstock that have been held in conditioning systems for extended periods. The nonfeeding larvae have a short larval phase (e.g., 7 days at 17°C for *Haliotis rubra* Leach and *Haliotis laevigata* Donovan). When larvae are ready for settlement they actively seek a suitable surface. In the natural environment, abalone larvae settle on coralline red algae (Shepherd & Daume 1996); however on farms the surface is typically vertical, spaced plastic plates that have been colonized by a variety of different algal species. Abalone aquaculture in most countries is dependent on cultured algae at least for the early life stages, to induce larval settlement and as a food source for postlarvae and juveniles, until formulated food is introduced into the growout system. As provision of algal supplies decline, the juveniles may be weaned onto formulated foods. They can be transferred to various land-based tanks or sea-based systems (Freeman 2001). In several countries around the world (e.g., South Africa) even the growout depends

solely on algae; macroalgae that are harvested from the ocean are fed to the abalone in specialist growout systems. A large component of the cost of producing juveniles is the provisioning of live food in a manner suitable for a grazing herbivore. This review examines the roles of bacteria, micro and macroalgae during the nursery phase of abalone aquaculture and emphasizes research conducted by the author with postlarval and juvenile *H. laevigata* and *H. rubra* in Australia. It complements earlier reviews by Roberts (2001) on larval settlement and by Kawamura et al. (1998c) on postlarval growth and survival by highlighting the applicability of bacteria and algae for commercial abalone hatcheries and nurseries. Their roles are considered in the context of the main areas of research undertaken to improve juvenile production efficiency: (1) presettlement larvae quality; (2) larval settlement; (3) dietary requirements for postlarvae and juveniles.

### Pre-settlement Larvae Quality

Previously wild abalone broodstock that feed on a range of macroalgae have been the main source of gametes for commercial abalone hatcheries. Selection of broodstock is mainly based on gonad size and appearance (Litaay & De Silva 2001), with abalone judged to be ready for induced spawning and have mature eggs based on the amount of swelling of the gonad. However, animal selection based on these criteria shows variable results in spawning success and produce offspring with large variability in larval and postlarval survival. More recently there has been greater commercial and research interest in conditioning captive and farmed broodstock using macroalgae or formulated foods (Grubert & Ritar 2003, Daume & Ryan 2004a, Freeman et al. this volume).

Lipids and protein in abalone eggs are known to fuel the development and metamorphosis of the larvae (Jaeeckle & Manahan 1989a, 1989b, Litaay et al. 2001). Nelson et al. (2002) demonstrated that variations in lipid content and fatty acid profile of the digestive gland coincided with variation in their macroalgal diets

and are related to seasonal temperature fluctuations. Biochemical variation in the diet may affect the composition of the eggs and ultimately larval performance. However studies of changes in biochemical composition such as fatty acids in abalone eggs are scarce. Litaay et al. (2001) demonstrated changes in biochemical composition during larval development. Recently, Daume and Ryan (2004a) showed high variability in proximate biochemical composition and fatty acid profiles of abalone eggs between hatches derived from conditioned and wild broodstock as well as between two consecutive spawning seasons. The relative proportions of some PUFAs in the broodstock diets were reflected in the eggs and varied between hatches of conditioned and wild broodstock, indicating that formulated diets designed to maximize growth rates are not necessarily adequate to maintain viable, high quality eggs and larvae from captive broodstock.

Other factors that can influence the quality and success of larval culture are opportunistic pathogenic bacteria that can bloom and cause deformities in and collapse of whole larval batches under potentially stressful commercial growing conditions. Many abalone hatcheries are using antibiotics like oxytetracycline prophylactically. Similarly they may be used in research projects. Roberts (2001) suggested using antibiotics to eliminate bacterial interference in settlement assay systems. Apart from the general problem of development of antibiotic resistant strains of bacteria in hatcheries, problems have been reported with certain antibiotics when used with abalone during larval rearing or settlement assays. Streptomycin at low doses of  $5 \mu\text{g mL}^{-1}$  was toxic to *Haliotis diversicolor* (Bryan & Qian 1998). Emitine caused abnormal loss of velum that could have been confused with metamorphosis (Fenteany & Morse 1993).

An experiment conducted to assess the effect of two antibiotics (Ampicillin and Kanamycin at  $50 \mu\text{g mL}^{-1}$ ) on the settlement of *H. rubra* revealed no difference in settlement rate between treated and untreated settlement substrate (Table 1). In this experiment 3 algal settlement substrata were tested (*Navicula cf. jeffreyi*, *Ulva lens*, *Sporolithon durum*) and compared with a negative control (plastic square of commercial settlement plate without any algal growth) all with and without antibiotics. The ratios of settlement rates between treated and untreated substrates did not change over time. In addition, the difference in settlement preferences between specific substrates remained the same regardless if antibiotics were used or not. The antibiotics were initially effective as indicated by the higher survival of swimming larvae (in water column) in control jars treated with antibiotics. However, the settlement rate was

not higher in the antibiotic treatment, indicating that unfit larvae might survive if treated with antibiotics but they do not settle successfully. This result questions the need and usefulness of antibiotics in abalone hatcheries. Further studies are needed to assess the effects of other antibiotics and earlier treatment with antibiotics (e.g., during larval rearing). However, alternatives like probiotics should be investigated to enhance larval survival safely.

Many antibiotics, including Kanamycin and oxytetracycline, work by inhibiting or interfering with the protein biosynthesis by targeting the bacterial ribosomes. The close similarity between bacterial and mitochondrial ribosomes makes the latter (present in all cells of the "treated" organisms) a potential target (Hart 2004). Inhibition of mitochondrial protein synthesis or injuries in mitochondria of the treated organism have occurred and can lead to various dysfunction; any cell type or tissue with a high aerobic energy requirement is more likely to be affected when this organelle is injured (Hart 2004). The effects of antibiotics on abalone larval settlement and postlarval performance however are not well understood. The knowledge we have from other systems, however, warrants extreme caution and highlights the danger of introducing other, potentially detrimental factors. These may not be obvious initially but may manifest themselves at later stages of larval or postlarval development.

#### Larval Settlement

The term "settlement" in this review describes the permanent attachment of abalone larvae to the substrate after shedding of the velum to complete metamorphosis. In the natural environment, abalone larvae, like many other invertebrate larvae, settle on coralline red algae. Daume et al. (1999a) revealed that settlement of *Haliotis laevis* larvae in response to three nongeniculate coralline red algae is species-specific. In that study the frequency of occurrence of epiphytic bacteria and diatoms was assessed on all coralline red algal species tested. However, no significant correlation was found indicating that the settlement induction is algal in origin. The authors concluded that bacteria and diatoms may influence the settlement response of abalone larvae but they are not the main driving force. Roberts (2001) referred to some of his unpublished work and stated that bacteria can induce abalone larval settlement but that the response is slow, taking 1 week to reach 50% metamorphosis. In contrast, very rapid settlement was reported in small-scale laboratory experiments through the use of the coralline red alga, *Sporolithon durum*, with the maximum rate

TABLE 1.

Percentage settlement of *Haliotis rubra* on different settlement substrates (*Ulva lens* and *Navicula cf. jeffreyi* and a negative control), with and without antibiotics, as well as *Sporolithon durum* (positive control) after 24, 48 hours, % settled and survived up to 1 week and % of larvae in water column after 1 week ( $n = 6 \pm \text{SE}$ ). Data are from Daume (2003).

Species	Antibiotics	% Settlement 24 Hours	% Settlement 48 Hours	% Survival Up to 1 Week	% in Water Column After 1 Week
<i>Ulva lens</i>	–	$30 \pm 8.1^a$	$35 \pm 7.6^a$	$12 \pm 1.5$	$0 \pm 0$
<i>Ulva lens</i>	+	$22 \pm 4.4^a$	$36 \pm 5.3^a$	$17 \pm 1.1$	$5 \pm 1.6$
<i>Navicula cf. jeffreyi</i>	–	$5 \pm 1.4^b$	$3 \pm 2.0^b$	$4 \pm 1.2$	$8 \pm 3.5$
<i>Navicula cf. jeffreyi</i>	+	$0.3 \pm 0.3^b$	$1 \pm 0.3^b$	$2 \pm 0.3$	$30 \pm 4.2$
Control	–	$0 \pm 0^b$	$1 \pm 0.6^b$	$0.5 \pm 0.3$	$3 \pm 1.1$
Control	+	$0 \pm 0^b$	$0.3 \pm 0.3^b$	$0.6 \pm 0.3$	$54 \pm 6.7$
<i>Sporolithon durum</i>	–	$39 \pm 3.7$	$50 \pm 4.6$	$16 \pm 2.6$	$0 \pm 0$

Means with different superscript letters are significantly different ( $P < 0.05$ ).



being reached after 24 h (Daume et al. 1999a) indicating that nongeniculate coralline red algae are strong settlement inducers. This result coincides with disproportional high numbers of recruits found on *S. durum* in the natural environment (Shepherd & Daume 1996).

Historically, benthic biofilms, consisting of bacteria and mixed diatom species growing on PVC settlement plates, have been used in abalone hatcheries worldwide to induce larval settlement. Diatoms, brought in by the incoming seawater, colonize clear plastic sheets arranged in commercial nursery tanks. This process is unpredictable and larval settlement rates can be low (1% to 10% of larvae) (Daume 2003). In both experimental and commercial systems, to achieve more control and consistency, films dominated by single algal species can be generated (Daume et al. 2000, Daume & Ryan 2004b). *H. rubra* did not respond to films of any diatom species tested, but settled on the nongeniculate coralline red alga *Phymatolithon repandum* (Daume et al. 1999b). In contrast, *H. laevisgata* settled comparably well on the diatom *Navicula ramosissima* and on the coralline *S. durum*. Roberts (2001) reviewed data on settlement cues including diatoms and other biofilms. Overall it is apparent that coralline red algae provide more consistent and reliable settlement cues, whereas settlement on diatoms can be highly variable. However, propagation of coralline red algae is not practical at a commercial scale.

Abalone hatcheries in Japan successfully settle abalone larvae (*Haliotis discus hannai*) on the green alga *Ulva lens* (Takahashi & Koganezawa 1988). *U. lens* is also suitable for enhancing settlement of both cultured southern Australian abalone species (*H. rubra* and *H. laevisgata*) (Fig. 1). Most abalone farms in Australia are now growing *U. lens* for that purpose (Daume et al. 2000, Daume et al. 2004, Daume & Ryan 2004b). The earlier study established settlement preferences of *H. rubra* for *U. lens* at laboratory scale whereas the later studies focused on commercial scale experiments. Both species (*H. rubra*, *H. laevisgata*) showed a clear preference for older rather than for younger *U. lens* (Table 2, Table 3) even with similar percentage cover, indicating that the developmental stage of the alga and not percentage cover *per se* is important in settlement induction (Table 3). Settlement was also significantly higher in the combined *U. lens* treatments (old and young) compared with 2 diatom treatments (*Navicula cf. jeffreyi* and *Cocconeis* sp. demonstrating the suitability of *U. lens* to improve the settlement of *Haliotis laevisgata* larvae on commercial scale (Table 3). No significant difference between high and low larval release densities was found with *H. rubra* in the nursery (Table 2) confirming earlier findings at laboratory scale with *H. laevisgata* larvae that settlement of abalone larvae is not gregarious when tested with larvae of the same batch (Daume et al. 1999a). In contrast, settlement was found to be gregarious in response to conspecific postlarvae as young as 7 days (Daume et al. 1999a) and older conspecific juveniles and adults and their grazing mucus is believed to be responsible (Seki & Kan-no 1981, Slattery 1992).

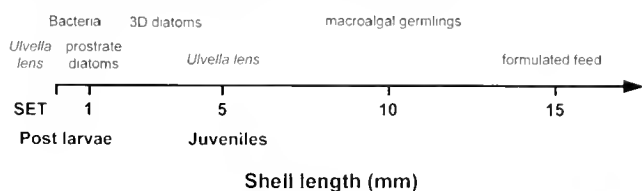


Figure 1. Sequence from settlement cue to potential food items proposed for Australian temperate abalone species in commercial farming systems, as they grow.

TABLE 2.

Percentage settlement ( $\pm$ SE) of *Haliotis rubra* in the nursery 3 days after larval release ( $n = 32$ ). Data from Daume et al. (2004).

Larval Density	<i>Ulva lens</i>	Per <i>U. lens</i> Treatment	Total per Tank
High	Old (18 days 31% cover).	31.9 $\pm$ 7.5	53.6 $\pm$ 5.8
	Young (4 days 57% cover).	21.7 $\pm$ 6.8	
Low	Old (18 days 31% cover).	44.0 $\pm$ 7.3	70.4 $\pm$ 8.7
	Young (4 days 57% cover).	26.4 $\pm$ 7.6	
Average			62.0

Recently alternative systems, to replace live algae as a means of settlement and growing postlarvae, have been proposed in Japan for *H. discus discus* and *H. diversicolor* (Stott et al. 2002, 2003, 2004a, 2004b). In the earlier studies, an alginate gel solution containing micro particulate diets was pasted onto settlement plates. In more recent studies settlement plates are sprayed with a solution of agar and one of the following: dried algal powder (*Spirulina platensis*, *Chlorella vulgaris*, *Undaria pinnatifida*), dried natural diatom powder, formulated diet and two different concentrations of  $\gamma$ -aminobutyric acid (GABA), each with and without antibiotics, and compared with negative (clean plates) and positive (living natural diatom biofilms). In both recent studies there was no significant difference in settlement rates between the microalgae powder treatments and the living natural biofilm but both supported significantly higher rates when compared with the negative control and GABA treatments (Stott et al. 2004a, 2004b). The authors demonstrated that pregrazing of plates by conspecific juveniles covered with microalgal powder/ agar solution enhanced larval settlement significantly (85% vs. 30% on grazed and ungrazed plates respectively). This system shows some potential, however mechanized and cost-efficient ways of spraying the plates need to be developed before it becomes viable commercially.

#### Dietary Requirements

Post-larval abalone feed on benthic diatoms (Kawamura et al. 1995) and the diatom film on plates also provides the food for growing postlarvae in commercial abalone nurseries. Commercial farms traditionally rely on mixed species of diatoms as a food source throughout the nursery period (settled larvae to 8–10 mm). The film is maintained through passive seeding (new cells are brought in with the incoming seawater), adding nutrients and manipulating the light intensity through shading. Without much control over composition and density of the biofilm species, the results are very inconsistent and often very poor. Isolating particular diatom species and growing them in monoculture before inoculating settlement tanks in the nursery affords greater control. This however has not been embraced by the industry and further investigations are needed to assess the effectiveness in larger scale systems. However, a significant bottleneck experienced by industry is the inability to maintain adequate food (both quantity and quality) on the plates particularly at later stages of the nursery phase. Growth rates of juveniles are influenced by the availability, digestibility and nutritional composition of the algae (Kawamura et al. 1998b, Roberts et al. 1999, Daume et al. 2003).

#### The Role of Bacteria in Postlarval Nutrition

Diatom cultures and biofilms developing on settlement plates are not axenic and the role of bacteria in early postlarvae feeding

TABLE 3.

Percentage settlement ( $\pm$ SE) of *Haliotis laevis* 3 days after larval release ( $n = 3$ ) when given a choice between 4 substrates. Data from Daume and Ryan (2004b).

Treatments	Old <i>U. lens</i> (8 weeks–97% cover)	Young <i>U. lens</i> (6 weeks–82% cover)	<i>Navicula</i> sp.	<i>Cocconeis</i> sp.	Total per Tank
% Settlement	61 $\pm$ 14	14 $\pm$ 1	7 $\pm$ 0.3	5 $\pm$ 0.5	87

and growth is poorly understood. Newly settled postlarvae ingest diatoms but are often not able to digest the cell contents. This suggests that bacteria and the extracellular material produced by the diatoms, present in the biofilm, are a significant source of nutrition for postlarval abalone (Fig. 1). Garland et al. (1985) reported that postlarval *H. rubra* ingested bacteria growing on the surface of coralline red algae. It has been suggested that bacteria may perform metabolic activities in the undeveloped gut of young postlarvae and are able to enhance the digestion efficiency of the host by supplying polysaccharolytic enzymes (Garland et al. 1985, Erasmus et al. 1997). Polysaccharolytic enzyme activity has been reported in day 17 *H. discus hawaii* postlarvae (Takami et al. 1998). Sawabe et al. (2003) detected the bacteria *Vibrio halioticoli* in the gut of *H. diversicolor aquatilis* and suggested that this bacterium may play a crucial role in converting alginate to acetic acid. As part of the alternative systems proposed by Stott et al. (2002, 2003, 2004a, 2004b), the authors observed that the growth of postlarvae *H. diversicolor aquatilis* fed a formulated diet was reduced when antibiotics were added and suggested that bacteria that assisted in digestion became limiting. In a later study they discovered that 5–10 times more bacteria (including *Vibrio* spp.) were present on plates sprayed with the agar/formulated diet solution. These bacteria could have provided a substantial food source to early postlarvae, which may have contributed to the significantly better growth rates on these plates 1 week after settlement (Stott et al. 2004b). The authors suggest that for recently settled postlarvae, bacteria might be a superior food source compared with diatom and abalone grazing mucus. All these studies indicate that bacteria are ingested and play an important role in early postlarval nutrition and health, but further studies are needed to elucidate their role and contribution.

#### Food Preferences for Postlarval Abalone

Worldwide, several studies have examined postlarval feeding and growth on different algal species (Ohgai et al. 1991, Ishida et al. 1995, Kawamura et al. 1998a, Roberts et al. 1999). Studies devoted to examining their feeding preferences and growth (Kawamura & Kikuchi 1992, Kawamura & Takami 1995, Kawamura et al. 1995, Matthews & Cook 1995, Kawamura 1996, Takami et al. 1997, Daume et al. 2000, Takami & Kawamura 2003) have shown that food requirements change as abalone grow (Fig. 1). Two to three weeks after settlement, postlarvae become responsive to the “digestibility” of the diatom strains and grow more rapidly on effectively digested strains (Kawamura et al. 1998a, 1998b). Postlarvae 0.8–2 mm in shell length grow ca 40–60  $\mu\text{m day}^{-1}$  on “digestible” diatoms and only ca 15–30  $\mu\text{m day}^{-1}$  on “indigestible” diatoms (Kawamura et al. 1998b). In addition, the diatom cell size, attachment strength, frustule’s strength and postlarval size can influence digestion. In a feeding trial covering the whole postlarval period, Roberts et al. (1999) showed that different diatom food species affected survival and growth. After day 17, postlarvae

grew faster on *Cocconeis scutellum* and *Cylindrotheca closterium*. Both species were most efficiently digested. Transitions in postlarval feeding preferences and growth performances on different algal species are reviewed in Kawamura et al. (1998c).

#### Alternative Food Sources for all Stages of Nursery Culture

The green alga *U. lens* has limited value as a food for growing postlarvae. Instead, cultured diatoms can be added after larvae successfully settle and start feeding. Seki (1997) reported that growth rates of postlarvae on *U. lens* were improved by the inoculation of cultured diatoms.

Recent studies showed that plates with a low cover of young germings of *U. lens* could be used for settlement induction of Australian abalone species (*H. rubra*, *H. laevis*) and followed with inoculation of the cultured diatom *Navicula* cf. *jeffreysi* to ensure sufficient food for the growing postlarvae (Daume et al. 2000, 2004, Daume & Ryan 2004b). The former study provided crucial information on early development of *H. rubra* and established that growth rates on several diatom species are significantly higher than on *U. lens* at laboratory scale (Fig. 2). In the more recent study, at commercial scale, the type of substrate on which larvae settled, light (which affected the food density) and the density of postlarvae all had very marked effects on growth (Daume et al. 2004). The results also suggest that early growth is important in determining later performance. Daume and Ryan (2004b) investigated settlement, growth, survival and size variability of the abalone *H. laevis* on commercial scale. Both growth rate and size variability increased over time until juveniles reached approximately 5 mm in shell length. Whereas postlarval abalone do not grow well on *U. lens* (Fig. 2), juvenile abalone (>3 mm in shell length) can consume *U. lens* and grow rapidly (80–110  $\mu\text{m day}^{-1}$ ) on this alga (Table 4).

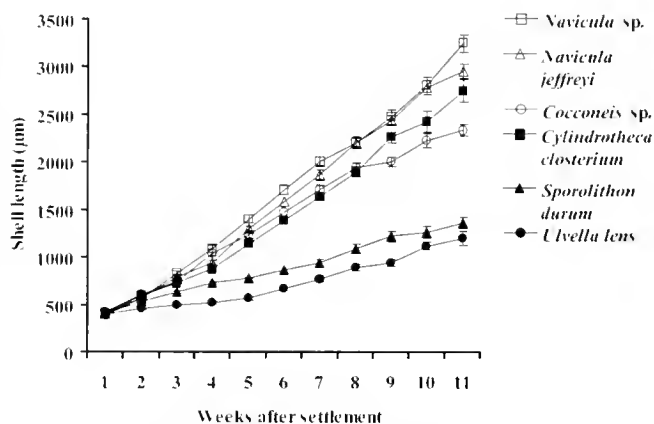


Figure 2. Early growth of *H. rubra* postlarvae feeding on different algal species. Vertical bars indicate standard error;  $n = 4$ . Data from Daume et al. (2000).

TABLE 4.

Daily growth-rates ( $\mu\text{m day}^{-1}$ ) of juveniles (*Haliotis rubra*) on plates 52 days after settlement and shell length (mm) 114 days after settlement (mean  $\pm$  SE). Data from Daume et al. (2004).

<i>U. lens</i>	Daily Growth Rate ( $\mu\text{m day}^{-1}$ )			Shell Length (mm)
	52–64 Days	64–94 Days	94–114 Days	114 Days
Old	79.4 $\pm$ 7.7	107.4 $\pm$ 4.2	82.8 $\pm$ 4.2	6.9 $\pm$ 0.2
Young	94.9 $\pm$ 8.4	115.3 $\pm$ 14.8	87.8 $\pm$ 8.2	7.4 $\pm$ 0.2

At later stages of the nursery phase (>5 mm in shell length), it becomes increasingly difficult to maintain adequate food on the plates and this is still regarded as a significant bottleneck for the industry. Recent investigations have indicated that sporelings of macroalgae like *Ulva* sp. may provide a suitable food source for juveniles (see Strain et al. this volume) (Fig. 1). Alternatively, chain forming diatoms, like *Delphineis*, offer a 3-D structure compared with the 2-D structure of nonchain forming prostrate attaching species, like *Navicula* spp. and thus providing more biomass for the growing juveniles (Fig. 1). Kawamura et al. (1995) reported growth rates of 48  $\mu\text{m day}^{-1}$  of *H. discus hamui* juveniles 1–2 mm in shell length, when feeding on the diatom *Achnanthes longipex*, which has a 3-D structure. More recently, Takami and Kawamura, (2003) found that juveniles 2.8–2.9 mm in shell length grew 100  $\mu\text{m day}^{-1}$  on this diatom species, which was comparable to growth rates achieved on juvenile sporophytes of the macroalga *Laminaria japonica*.

#### Biochemical Composition and Nutritional Value of Algal Diets

The biochemical composition of microalgae, and therefore their nutritional value to herbivores varies between species (Brown et al. 1996) and is greatly affected by harvest stage, light intensity (Thompson et al. 1993, Brown et al. 1996), nutrient concentrations (Fábregas et al. 1996, Fábregas et al. 1998) and culture methods (Otero & Fábregas 1997). It is known that the biochemical composition of algae can be altered by changing the growing conditions (e.g., Otero & Fábregas 1997, Thompson et al. 1993, Brown et al. 1996). When microalgal cultures are grown in nitrogen-limited media, the protein content of the cells decreases (Enright et al. 1986, D'Souza & Kelly 2000, Daume et al. 2003). Daume et al. (2003) showed previously that juvenile *H. rubra* grew faster when feeding on the diatom *Navicula* cf. *jeffreysi* that was cultured in a

higher nitrate medium. Searcy-Bernal et al. (2003) found that recently settled *H. fulgens* postlarvae grew and survived better under low light (6  $\mu\text{E}$ ) conditions, whereas a lower number of cells of the diatom *Navicula incerta* were available in the lower light treatment. The authors suggested that oxygen supersaturation in the boundary layer, particularly in high-density diatom films at high light levels (75  $\mu\text{E}$ ), could have caused high mortality in this treatment. In another study, the influence of light intensity on two diatom species (*Navicula* cf. *jeffreysi*, *Cocconeis* sp.) as a food for juvenile *H. laevigata* (3–4 mm in shell length) was tested (Watson et al. 2004). In contrast to *N.* cf. *jeffreysi*, growth of *Cocconeis* sp. was not inhibited at lower light levels making it a good candidate for culture in shaded nursery systems. Light was more influential in juvenile grazing behavior (photophobic) than food availability. Watson et al. (2005) examined the combined effect of manipulations in light intensity and nitrate concentrations on the nutritional value of the diatom *Navicula* cf. *jeffreysi* when fed to juvenile abalone (*H. laevigata*). Under high light conditions *Navicula* cf. *jeffreysi* was lower in protein and higher in carbohydrates and fat. Juveniles grazed larger numbers of diatom cells when the protein content was low, possibly compensating for the lower protein levels. The authors reported elevated pH levels in higher light treatments and suggested that this could have caused high mortality. These studies indicate that changes in light intensity and nitrate concentration, under which the diatom species are cultured, can have a dramatic effect on growth, grazing rates and particularly survival of postlarval and juvenile abalone. This emphasizes the need for selecting the right light and nutrient level to achieve high value food and conditions for optimal growth and survival of juvenile abalone in commercial nurseries.

This study reviewed three main areas of abalone research associated with abalone hatchery and nursery production. Further studies are needed to find alternatives, such as probiotics, to the use of antibiotics in abalone hatcheries. Alternative cost effective foods, for broodstock and for the latter stage of the nursery still need to be found that will increase larval quality and allow abalone farmers to keep animals on the plates longer and thus reduce weaning mortality.

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## EFFECT OF THE POLLUTANTS LEAD, ZINC, HEXADECANE AND OCTOCOSANE ON TOTAL GROWTH AND SHELL GROWTH IN THE AKOYA PEARL OYSTER, *PINCTADA IMBRICATA*

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**ABSTRACT** Pearl oysters (*Pinctada imbricata*) were held in the laboratory and exposed to various levels of the heavy metals lead and zinc and the aliphatic hydrocarbons hexadecane and octacosane for 2 months. Individual oysters were followed over the course of the experiment, allowing specific calculation of total oyster growth (wet weight) and shell growth. Significant reductions in total oyster growth were observed when oysters were exposed to high concentrations (270 µg L<sup>-1</sup>) of either zinc or lead. Exposure to the aliphatic hydrocarbons had no effect on total oyster growth. High concentrations of lead completely halted shell growth, the first demonstration of pollutant induced cessation of biomineralization in pearl oysters. Conversely, exposure to moderate levels of lead and the long-chain hydrocarbon octacosane resulted in significant increases in shell width growth. The results from this study indicate that *P. imbricata* is relatively tolerant of the selected pollutants and could be deployed within a remediative context in moderately polluted coastal areas.

**KEY WORDS:** *Pinctada*, oyster, pollution, biomineralization, pearl, metals, shell, aquaculture

### INTRODUCTION

The Akoya pearl oyster, *Pinctada imbricata* (Röding 1798), has a broad global distribution and is found in temperate and tropical waters. This species has been farmed in Japan for over 100 y for its small yet lustrous pearls. But Japanese production of high quality Akoya pearls has fallen from 118,000 kg in 1993 to 63,000 kg in 1996, with continuing production declines (Miyazaki et al. 1999). This decline has in part been attributed to deteriorating water quality (Tomaru et al. 2001). It is well known that bivalve molluscs accumulate many pollutants within their tissue and shell, a fact that has led to their use as biomonitors of hydrocarbons (Sericano et al. 1995) and heavy metals (Bourgoin 1990, Phillips & Rainbow 1993) pollution in marine and estuarine waters. Pearl oysters are no exception to this, and have been used as biomonitors of heavy metals (Bou-Olayan et al. 1995) and hydrocarbons (Fowler et al. 1993). Whereas it is known that pearl oysters accumulate hydrocarbons and heavy metals in their tissues on exposure and that these compounds can exert toxic effects on other bivalve molluscs (Kennedy et al. 1996), there is little information regarding the tolerance of pearl oysters to pollutants.

The tolerance of *P. imbricata* to particular pollutants is of further interest because pearl aquaculture has recently been proposed as a coastal remediation technology (Gifford et al. 2004). Pearl oysters have a high filtration rate, concentrate pollutants and nutrients within their tissues, and they yield a valuable product that is not bound for human consumption. However, the success of a pearl oyster remediation system would rely on the profitability of pearling operations to make the coastal remediation commercially viable. Therefore, it is necessary to investigate the oyster's tolerance limits, for both general pearl oyster health and pearl quality.

Relatively little is known about the effects of pollutants on shell biomineralization in molluscs. In the Pacific oyster, *Crassostrea gigas*, exposure to tributyltin results in the production of a gelatinous substance within the shell and shell deformity (Alzieu et al. 1986), whereas exposure of the mussel, *Mytilus californianus*, to barium results in abnormal shell calcification (Spangenberg &

Cherr 1996). In one of the few studies to investigate the effects of dietary pollutant exposure on shell biomineralization, high (500 µg g<sup>-1</sup>) dietary concentrations of lead resulted in a 25% reduction in shell mass in juvenile garden snails (*Helix aspersa*), yet shell size was unaffected by lead (Beeby et al. 2002). Similarly, transplanted *Crassostrea gigas* exposed to high concentrations of cadmium, copper and zinc in Chesapeake Bay had significantly thinner shells than control oysters (Frazier 1976). High concentrations of cadmium have also been shown to inhibit shell growth in *Crassostrea virginica* (200 µg L<sup>-1</sup>, Shuster & Pringle 1969) and *Mytilus edulis* (500 µg L<sup>-1</sup>, Stureson 1978). Exposure of *C. gigas* to lead for 4 months led to significant differences in the amino acid profile of the shells (Almeida et al. 1998). Shell length growth in *M. edulis* is significantly reduced by exposure to the heavy metals zinc, mercury, copper, and cadmium but not lead and nickel (Strömberg 1982), and various hydrocarbon mixtures (Strömberg 1986, Strömberg et al. 1986). Given that high levels of pollutants alter shell production in many species of molluscs, it is possible that exposure to pollutants could alter shell growth in pearl oysters.

This study investigates the effects of pollutants on total growth and shell growth in pearl oysters. The oysters were exposed to either the essential metal zinc or the nonessential metal lead. These metallic pollutants are both common estuarine pollutants arising from urban, industrial and agricultural applications. Oysters were also separately exposed to either of the aliphatic hydrocarbons hexadecane and octacosane, common estuarine pollutants arising from urbanization and recreational and industrial boating. Furthermore, the hydrocarbons were chosen to represent differing hydrophobicity, which in turn has resulted in differing compartmentalization of pollutants between the shell and soft tissue of molluscs in previous work in our laboratory (Walsh et al. 1995). It was hypothesized that high concentrations of lead, zinc, hexadecane and octacosane would reduce both total and shell growth in the Akoya pearl oyster.

### MATERIALS AND METHODS

#### Experimental Design

The experiment was conducted at the NSW Fisheries Port Stephens Fisheries Centre from June to August 2003 according to

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the protocols of the American Society for Testing and Materials (ASTM) E 729-96 (1996) for static renewal tests. Fifty-one individually aerated 8 L aquaria were divided across two large water baths maintained at 22°C for the course of the experiment. Oysters used were from the same spawning batch from the NSW Fisheries oyster hatchery. Each aquarium contained a single oyster, allowing individual changes in oyster physiology to be monitored. After a 48-h acclimatization period and byssal attachment to the wall of the aquarium, exposure to the pollutant commenced. For the metal treatments, exposure was through the addition of sufficient  $\text{Pb}(\text{NO}_3)_2$  or  $\text{ZnSO}_4$  solution calculated so as to give final concentrations of background +10, 30, 90, 270  $\mu\text{g L}^{-1}$  of Pb and Zn. For the aliphatic hydrocarbons, sufficient pollutant dissolved in chloroform (at a concentration so as to ensure that the maximum concentration of chloroform in any aquaria was 0.2 ml  $\text{L}^{-1}$ ) was added to give the required concentrations of background +30, 90, 270, 810  $\text{ng L}^{-1}$ . The chloroform/hydrocarbon pollutant stocks were stored at -15°C and the metal solutions were stored at 4°C for the duration of the experiment.

Water was changed thrice weekly and the oysters were fed daily a mixture of the algae *Chaetoceros muelleri* and Tahitian *Isochrysis* aff. *galbana* and *Pavlova lutheri*. There were three replicate aquaria for each treatment. A control treatment with three replicates was also run. Replacement water was heated to 22°C prior to water changes and the salinity and temperature were monitored daily. Temperature and salinity throughout the experiment remained within the range  $22 \pm 0.5^\circ\text{C}$  and  $33 \pm 0.5 \text{ g kg}^{-1}$ , respectively.

#### Parameters Monitored

Before commencing, excess moisture was drained from the surface of the oysters and the total oyster mass was determined to the nearest 0.1 g, then shell length and shell width measured to the nearest mm using vernier calipers. After 2 months, the same parameters were measured.

#### Statistical Analysis

Differences in shell length, shell width and total oyster mass were analyzed using 1-way ANOVA. Normality and homogeneity of variance were verified using Levennes test and via graphing residuals, and the data were natural log transformed where necessary. The significance level was taken at  $P < 0.05$ . Posthoc analysis was conducted with pairwise Tukey HSD test. For the Gaussian correlation analyses, as well as testing for normality and homogeneity of variance, a power analysis was also conducted. All statistical analyses were carried out using SPSS v.10.1 and figures were compiled using SigmaPlot v 6.

## RESULTS

#### Total Oyster Growth (Increase in Wet Weight)

No oyster mortalities occurred during the experimental period and all oysters increased in total wet weight over the 2-month period. Oysters cultured in the control treatment increased total wet weight by, on average ( $\pm\text{SE}$ ),  $45 \pm 8\%$ .

Exposure to the higher concentrations of zinc and lead resulted in reductions in oyster growth as compared with control oysters (Fig. 1a, b). Oysters exposed to the highest experimental lead treatment had significantly reduced growth ( $F = 3.94$ ,  $P = 0.036$ , Fig. 1a), compared with control oysters. Whereas there was no

significant difference in the total growth of oysters exposed to the highest zinc treatment and control oysters, a significant decrease in total growth was observed for the 270  $\mu\text{g L}^{-1}$  zinc treatment compared with the 30  $\mu\text{g L}^{-1}$  treatment ( $F = 5.14$ ,  $P = 0.016$  Fig. 1b).

Exposure to the aliphatic hydrocarbons hexadecane and octacosane at 30–810  $\text{ng L}^{-1}$  did not reduce total body mass after 2 months (Fig. 1c, d).

#### Shell Growth

Exposure of the oysters to 270  $\mu\text{g L}^{-1}$  of lead significantly reduced shell length growth ( $F = 5.14$ ,  $P = 0.016$  Fig. 2a) as compared with the control treatment. The mean length of shells actually decreased by  $2 \pm 1\%$  (mean  $\pm$  SE) because of shell weakening at the distal margin. Visually, shells from oysters cultured in the high lead treatment lacked the imbricate processes at the distal margin evident in oysters grown in other treatments, indicating shell stress (Fig. 3). Although there was no impact on the measured shell length in the 90  $\mu\text{g L}^{-1}$  treatment, Figure 3 shows that these oysters also lacked the imbricate processes. These two highest lead concentrations resulted in shells that were weaker and more brittle than control oysters. Exposure to zinc did not significantly affect shell length growth over the 2-month period (Fig. 2b). Exposure to hexadecane and octacosane at 30–810  $\text{ng L}^{-1}$  did not have any significant effects on shell length (Fig. 2c, d).

Shell width growth was significantly greater in oysters exposed to 30 and 90  $\mu\text{g L}^{-1}$  lead, yet significantly decreased in oysters exposed to 270  $\mu\text{g L}^{-1}$  lead compared with control treatments ( $F = 4.6$ ,  $P = 0.02$ , Fig. 4d). Exposure to the longer chain hydrocarbon, octacosane, also resulted in increased shell width growth (Fig. 4d), but no significant differences to shell width were observed for oysters exposed to zinc and hexadecane (Fig. 4b, c).

#### Relationships Between Concentration of Pollutants and Shell Growth

For lead, zinc and octacosane, clear associations were apparent between the concentration of the pollutant and the percentage increase in shell width (Fig. 5). Moderate additions of these pollutants above the background level caused increases in the shell width growth. However, exposure to the higher experimental treatments resulted in reduced levels of shell width growth. For hexadecane, there was no relationship between pollutant concentration and shell width growth.

In contrast to shell width, whereas high concentrations of lead inhibit shell length growth, there was no relationship between concentration of pollutant and shell length growth for all 4 experimental treatments.

## DISCUSSION

Heavy metals inhibit growth in a variety of mollusc species (Manley et al. 1984, Wikfors et al. 1994, Din & Ahamad 1995, Keppler & Ringwood 2001). In this study, total oyster mass was reduced in *P. imbricata* oysters exposed to high concentrations of lead and zinc. The dose response was not linear, with no obvious effects on oyster growth at 10–90  $\mu\text{g L}^{-1}$  for lead and zinc; this indicates that *P. imbricata* is relatively tolerant of the individual effects of lead and zinc, at least up to concentrations of approximately 90  $\mu\text{g L}^{-1}$ . Effects on total oyster mass were observed at the higher concentration (270  $\mu\text{g L}^{-1}$ ). Typically, the concentration of dissolved lead in low-moderately human impacted estuaries is below 10  $\mu\text{g L}^{-1}$  (Barnes et al. 1982, Dassenakis et al. 1997). As



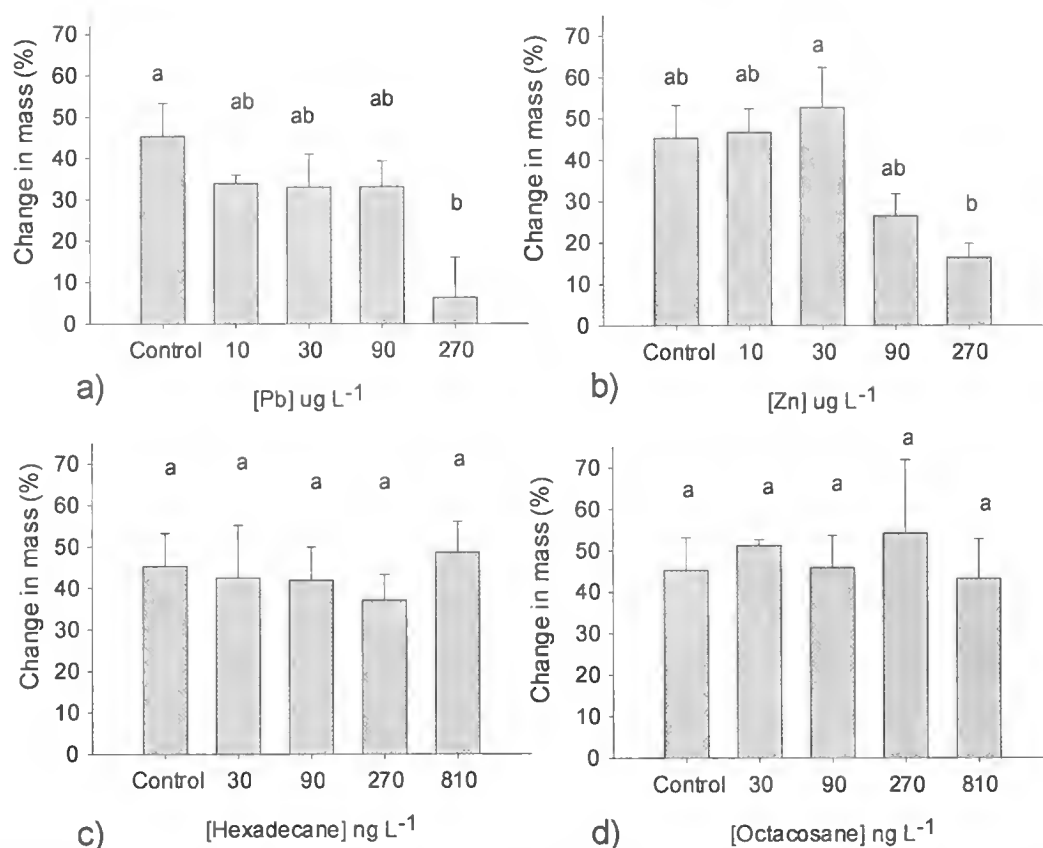


Figure 1. Mean change in mass (%) of *Pinctada imbricata* cultured under five different concentrations of lead, zinc, hexadecane and octacosane. Treatments identified as statistically similar are denoted by identical letters as determined by Tukey HSD pairwise comparison test. Error bars show SE ( $n = 3$ ).

such, this study provides important information on the zinc and lead tolerance limits of the commercially important Akoya pearl oyster. However, caution is necessary in extrapolating these laboratory results to field situations, because possible synergistic or antagonistic effects among pollutants may exist. Furthermore, the potential for size dependent changes of metal impacts on oyster health must also be considered.

Exposure to either of the aliphatic hydrocarbons used in this study did not significantly effect total oyster growth. Organic contaminants are known to adversely affect bivalve molluscs in a variety of ways including reduced feeding (Widdows et al. 1990), induction of stress proteins (Cruz-Rodríguez & Chu 2002), inhibition of oogenesis (Chu et al. 2000) and increased respiration (Widdows et al. 1990). However, most of these studies involve either halogenated hydrocarbons, such as polychlorinated biphenyls (PCB), or polycyclic aromatic hydrocarbons (PAH), with very little toxicological information regarding aliphatic hydrocarbons available. The results from this study indicate that dissolved concentrations of aliphatic hydrocarbons in the high  $\text{ng L}^{-1}$  range do not impact total growth of *P. imbricata*.

Shell structure of *P. imbricata* exposed to  $270 \mu\text{g L}^{-1}$  lead was visibly altered, with a complete lack of imbricate processes (frills) around the shell margin. Little information exists on the effects of pollutants on shell growth (Shuster & Pringle 1969, Frazier 1976, Strömberg 1982, Almeida et al. 1998, Beeby et al. 2002), and this is the first study to investigate the effects of pollutants on biomineralization in an economically important pearl oyster. Lead exposure has previously been shown to reduce shell thickness in the

common garden snail *Helix aspersa* (Beeby et al. 2002), and alter amino acid composition of the shell of *Crassostrea gigas* (Almeida et al. 1998). In our study, shell growth was completely impeded by exposure to  $270 \mu\text{g L}^{-1}$  of lead. Shuster and Pringle (1969) reported complete inhibition of shell growth by *Crassostrea virginica* exposed to similar concentrations of cadmium, whereas Strömberg (1982) observed cessation of shell growth in *Mytilus edulis* when exposed to copper and mercury. These results could be because of reduced activity of the enzyme carbonic anhydrase, an enzyme essential for shell and pearl production (Wilbur & Jodrey 1955, Freeman 1960, Miyamoto et al. 1996). Lead has previously been shown to inhibit levels of carbonic anhydrase in both anemones and corals (Gilbert & Guzman 2001), cadmium inhibits carbonic anhydrase in estuarine crabs (Vitale et al. 1999, Skaggs & Henry 2002) and eels (Lionetto et al. 1998), whereas silver, copper and zinc all reduce levels of carbonic anhydrase in crabs (Skaggs & Henry 2002).

Whereas high concentrations of lead significantly reduced shell growth, exposure to 30 and  $90 \mu\text{g L}^{-1}$  of lead had the opposite effect, significantly increasing growth in shell width. Whereas active metal incorporation into shell matrix has previously been described (Bertine & Goldberg 1972, Sturesson 1976, 1978, Al-Aasm et al. 1998), this is the first reported case of an increase in shell growth in response to moderate metal challenge. Exposure to octacosane also resulted in a significant increase in the growth of shell widths. In contrast, hexadecane did not significantly increase shell width growth. These results complement the earlier work of Walsh et al. (1995), who observed that longer chain hydrocarbons

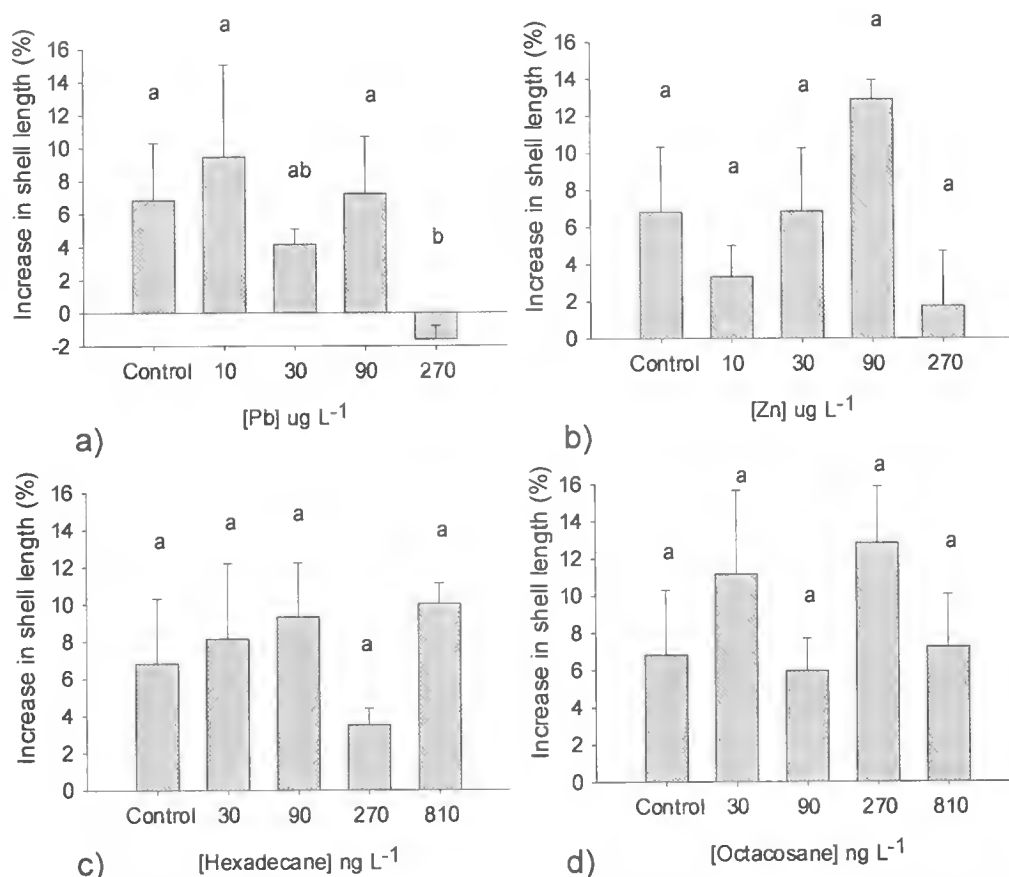


Figure 2. Mean change shell length (%) of *Pinctada imbricata* cultured under five different concentrations of lead, zinc, hexadecane and octacosane. Treatments identified as statistically similar are denoted by identical letters as determined by Tukey HSD pairwise comparison test. Error bars show SE ( $n = 3$ ).

(those compounds resistant to cellular degradation systems) preferentially accumulated in the shell of the gastropod *Austrocochlea constricta*, whereas shorter chain hydrocarbons were preferentially accumulated in the soft tissue of the organism. These authors proposed that the smaller aliphatic hydrocarbons might be more easily detoxified via cellular enzymatic systems within the soft tissue, whereas more hydrophobic and recalcitrant compounds could be actively partitioned into the shell matrix and thereby removing them from metabolically active tissue (and subsequently the food chain). The observed gaussian response of shell width to three of the four pollutants (Fig. 4) tested was consistent with this theory, although this result is highly dependent on the growth of

control oysters and in this experiment the control group consisted of only 3 oysters. Further studies are continuing to investigate the capacity of these animals to partition heavy metal and the more hydrophobic organic pollutants into the shell matrix.

In contrast to the results of this study, Strömberg et al. (1986) observed significant reductions in shell growth of the mussel *M. edulis* exposed to high concentrations of a microencapsulated mixture of n-alkanes. Concentrations used in our study were, however, approximately 1,000 times less than those used by Strömberg et al. (1986) and were the likely cause of the observed differences. Interestingly, Strömberg et al. (1986) found no difference in the effect of the aromatic and the n-alkane hydrocarbon mixtures on shell growth. Typically, aromatics are viewed as being more toxic than aliphatic hydrocarbons (Anderson et al. 1974). Given that very little is known about the effects of hydrocarbons on shell production (Strömberg 1986, Strömberg et al. 1986) further research in this area is clearly warranted.

Recently, pearl aquaculture has been proposed as a coastal bioremediation technology (Gifford et al. 2004). This is because of the fact that pearl oysters have high filtration rates (Pouvreau et al. 1999), concentrate pollutants within their tissue and shell (Al-Madfa et al. 1998, Bou-Olayan et al. 1995), have a high protein content (Suzuki 1957, Numaguchi 1995), are found native in many areas of the world (Colgan & Ponder 2002) and the highly valued pearl product is not bound for human consumption. Because the mechanism of pearl production is similar to that of shell production in the oyster, any effects observed on shell growth would likely be mirrored in pearl formation. Therefore, the inhibition of

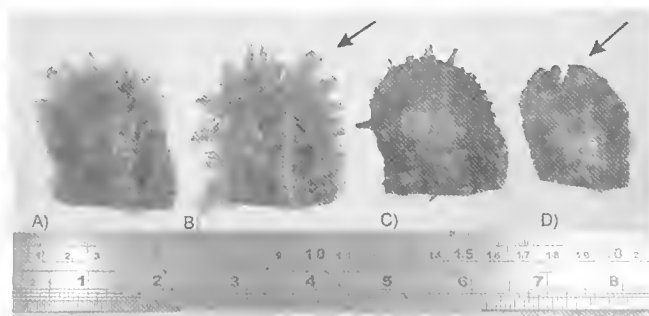


Figure 3. Oysters cultured under different concentrations of lead; a)  $10 \mu\text{g L}^{-1}$  b)  $30 \mu\text{g L}^{-1}$  c)  $90 \mu\text{g L}^{-1}$  and d)  $270 \mu\text{g L}^{-1}$ . Note the imbricate processes (marked in b) are absent around the shell margins for the oysters cultured under 90 and  $270 \mu\text{g L}^{-1}$ .

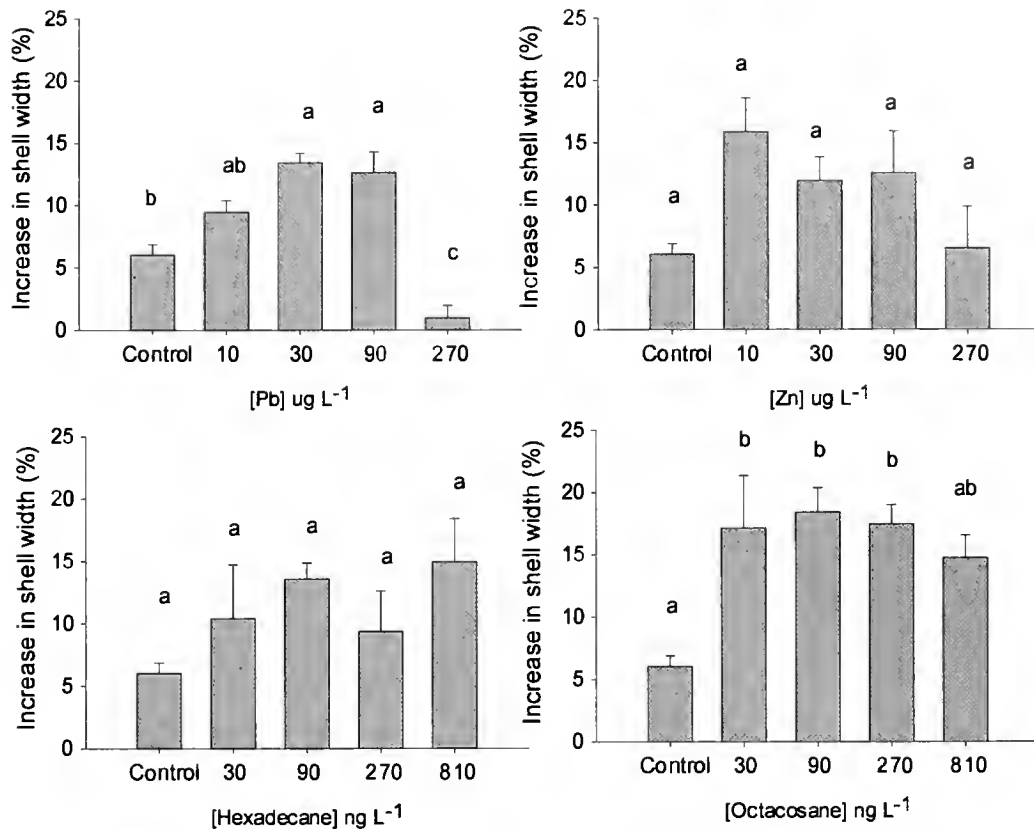


Figure 4. Mean change in shell width (%) of *Pinctada imbricata* cultured under five different lead, zinc, hexadecane or octacosane treatments. Treatments identified as statistically similar are denoted by identical letters as determined by Tukey HSD pairwise comparison test. Error bars show SE ( $n = 3$ )

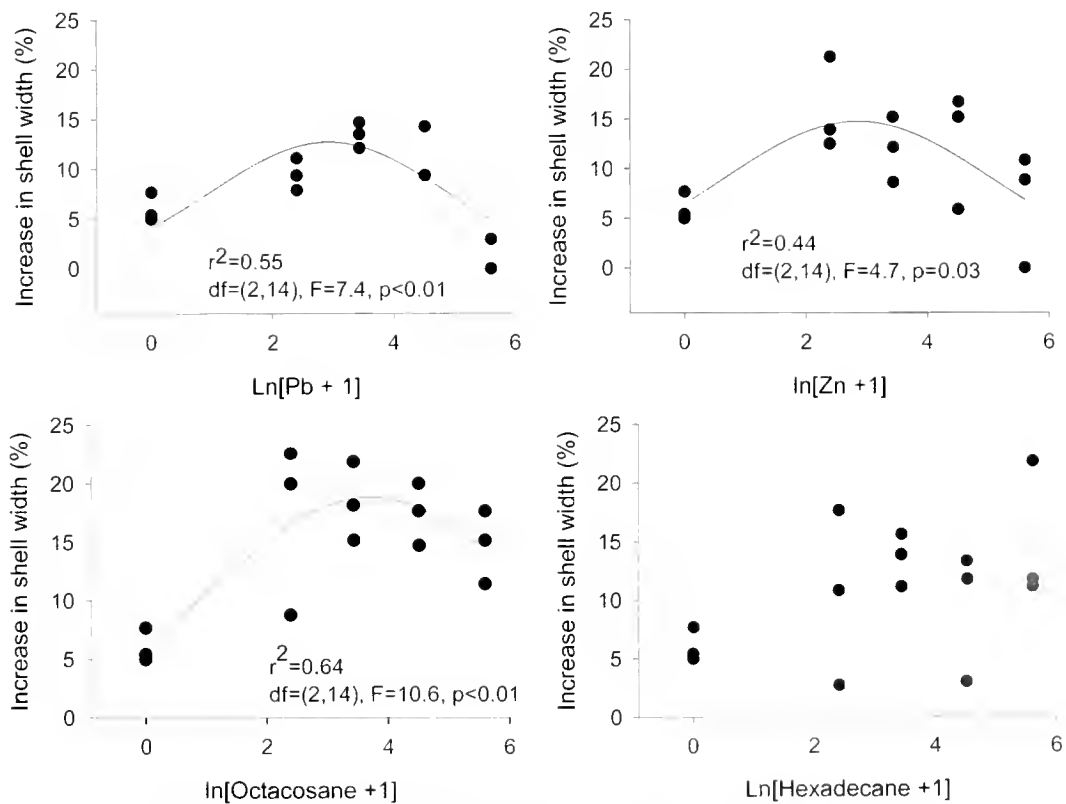


Figure 5. Relationships between concentration of pollutant and oyster shell width.

shell growth by high ( $270 \mu\text{g L}^{-1}$ ) concentrations of lead in this study demonstrates that pearl aquaculture would be unlikely to succeed in areas suffering from high concentrations of dissolved lead. However, moderate concentrations ( $10\text{--}90 \mu\text{g L}^{-1}$ ) of dissolved lead had the opposite effect, stimulating shell width growth. Furthermore, whereas high ( $270 \mu\text{g L}^{-1}$ ) zinc concentrations did not significantly effect shell growth, a finding similar to Mai et al. (2003) who found that dietary zinc did not effect biomineralization in the abalone *Haliotis discus hannai*, they did reduce total oyster growth. Therefore, pearl aquaculture would unlikely succeed in areas with high dissolved zinc concentrations. The concentrations of aliphatic hydrocarbons used in this study did not negatively affect any of the measured oyster parameters, and in fact stimulated shell width growth at  $30\text{--}270 \text{ ng L}^{-1}$ . Therefore careful evaluation of potential sites would be required to balance metal remediation requirements and pearl quality/oyster health outcomes.

### CONCLUSION

Exposure to high ( $270 \mu\text{g L}^{-1}$ ) concentrations of dissolved lead reduced total oyster growth in *P. imbricata*. Importantly, high ( $270$

$\mu\text{g L}^{-1}$ ) concentrations of lead significantly reduced shell growth and altered the visible appearance of the shell, the first demonstrated case of pollutant-impeded biomineralization in pearl oysters. However, intermediate concentrations ( $10\text{--}90 \mu\text{g L}^{-1}$ ) of lead and zinc actually stimulated shell width growth. Exposure to the aliphatic hydrocarbons hexadecane and octacosane had no effect on the total oyster growth. However, exposure to moderate ( $30\text{--}270 \text{ ng L}^{-1}$ ) concentrations of the long chain hydrocarbon octacosane significantly increased shell width growth. The results of this study demonstrate the general tolerance of *P. imbricata* to the pollutants lead, zinc, hexadecane and octacosane.

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## THE ROE'S ABALONE FISHERY NEAR THE PERTH METROPOLITAN AREA, WESTERN AUSTRALIA

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**ABSTRACT** The recreational Roe's abalone fishery is concentrated on easily accessible reefs that adjoin metropolitan Perth in Western Australia. There is a restricted recreational fishing season of 1.5 h a morning, for six consecutive Sundays in November and December each year. The reefs near Perth are also the focus of the commercial Roe's abalone fishery in Western Australia. The recreational catch and effort for each 10 nautical mile section of the Perth fishery is estimated from a creel survey with instantaneous counts, aerial surveys and interviews with fishers, from 1997–2003. Most of the recreational and commercial catches were taken from small areas of the fishery, with approximately 87% of the recreational catch coming from two 10 nm sections, and 98% of the commercial catch coming from these two and one additional 10-nm section. An annual quota of 36 t limits the commercial catch, whereas estimates of the recreational catch increased from 33–51 t between 1997 and 2003. Recreational catch and effort estimates from 1999 on were corroborated by an independent telephone survey, with independent estimates falling within the 95% confidence intervals.

**KEY WORDS:** Roe's abalone, *Haliotis roei*, recreational catch and effort, commercial catch, volunteer, creel survey

### INTRODUCTION

Roe's abalone, *Haliotis roei*, is distributed between the central Victorian coast and Shark Bay on the west coast of Western Australia (WA) (Shepherd 1973). It occupies the low intertidal and shallow subtidal areas of rocky reef adjoining the coast in south-western Australia (Shepherd 1973), and grows to a maximum size of about 120 mm (Wells & Keesing 1990). In the waters near Perth WA, Roe's abalone occupy the low intertidal and subtidal limestone reefs of the mainland and offshore islands. It also occurs on some offshore reefs topped by wave-cut platforms at about low tide level. In areas of favorable habitat, Roe's abalone attains densities approaching 200 m<sup>-2</sup> (Hancock 2004). Densities are highest at the seaward edge of the reef platform because of the settlement of the recruits in this habitat. Densities drop to low levels by about 1.5 m depth on the subtidal cliff at the seaward edge of the reef. Densities decline more gradually shoreward from the outer edge of the reef platform. The mean size of abalone tends to increase subtidally and shoreward from the seaward edge of the reef platform.

WA is the only state with a Roe's abalone fishery, but a feasibility study has been completed in South Australia (Preece et al. 2003). Commercial abalone fishing in the Perth area began in the early 1960s, but was small scale until 1970 when an influx of divers from other states caused a rapid increase in catches (Joll & Penn 1992). In 1970 the first of a complex array of area closures and restrictions on fishing time and daily catch were introduced for the commercial fishery in the Perth area. The Perth fishery is currently described in a series of sections of 10 min of latitude, (i.e., 10 nautical mile [nm] sections). For the commercial sector the Perth Roe's abalone fishery extends from the mouth of the Moore River in the north, to Cape Bouvard to the south (sections 36–43, Fig. 1). From 1992 to 2002 the recreational fishery extended approximately 30 nm further north, to Wedge Island (sections 33–43, Fig. 1). The commercial sector is excluded from fishing in the area around Rottnest Island, and the Cottesloe area of the mainland

coast. These areas remained open to recreational fishers until the closure of Cottesloe in 2003. Rottnest Island was not included in this study, and is not considered to be an important area of the fishery. The area around Penguin Island was closed to recreational fishers from 1996 to 1999, but remained open to some commercial fishing.

Since the summer of 1990 to 1991 the commercial catch from the Perth fishery has varied from 17.7–46.1 t whole weight, compared with a total catch from the state of approximately 108–119 t. During 1997 regulation of the commercial harvest of abalone from the Perth fishery was changed from mainly input-controlled to output-controlled by the removal of restrictions on allowable fishing times, and the introduction of quotas. Quotas were set at 3 t for each of the 12 commercial fishers (36 t total quota), for the period from October 1997 to September 1998 inclusive, reported here as the 1997 season. To align the quota periods for the different abalone managed areas around the state, the Perth season was adjusted to an April to March quota period.

Interest in the recreational fishery increased steadily from the early 1970s. By the early 1980s increasing fishing pressure had led to localized stock depletions and escalating conflict between the two user groups, resulting in a closure of the majority of the fishery for the 1983 season (Wells & Keesing 1989). Since the commencement of fishing in 1984, there has been a series of restrictions on both sectors, including the introduction of a recreational fishing licence in 1992. Since 1995 the season has consisted of 6 Sunday mornings from 7:00 AM to 8:30 AM, starting on the first Sunday in November. The minimum legal size of abalone taken by the recreational sector is 60 mm, with a daily limit of 20. The majority of fishers wade on the reef platform to search for abalone, with a small proportion snorkeling adjacent to the platform. The use of compressed air to collect abalone from the subtidal areas by recreational fishers is not permitted.

Australian abalone fisheries, including the *H. roei* fishery, are currently considered to be operating at sustainable levels; however globally there are cases where high exploitation levels have been a primary cause of the collapse of major fisheries. Notable instances include the collapse of fisheries in California (Tegner et al.

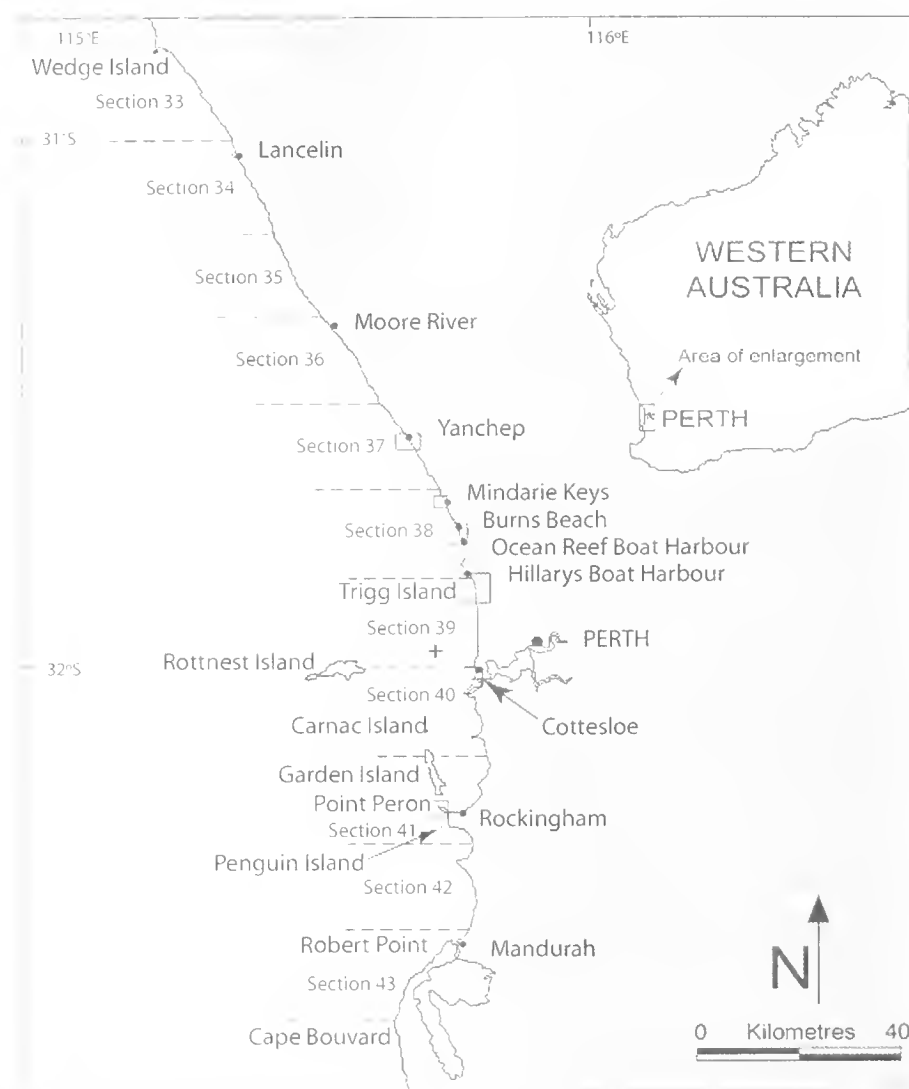


Figure 1. Map of Western Australia, showing the area of the Perth abalone fishery (inset), subdivided into 10 nm sections (enlargement). Boxes indicate the portions of the fishery designed as high usage zone, enlarged in Fig. 2. + indicates the location of the wave recording station.

1992, Parker et al. 1992, Karpov et al. 1998) and Mexico (Guzmán del Próo 1992), with more recent concern for the sustainability of the South African fishery (Tarr 2000, Tarr et al. 2000).

Recreational fisheries are increasingly recognized as making up an important component of the total fishing harvest of exploited nearshore marine populations (Kearney 1995). Although commercial catches are often carefully recorded, recreational catches are not as easily quantified. Consequently there is an emphasis on measuring the recreational catch component of many fisheries. The aim of measuring the recreational catch and effort has generally been: (1) to quantify an important impact on a fished stock, in concert with commercial catch; (2) to address issues of fishery sustainability and (3) to establish a basis for informed debate regarding resource sharing between interest groups (Kearney 1995). Surveys of recreational abalone catch and effort conducted in New South Wales (Worthington et al. 1998) and Tasmania (Lyle & Smith 1998), in New Zealand (Teirney et al. 1997, Bradford 1998) and South Africa (Anon 1998, 1999), have generally concluded that catch and effort are increasing where management restrictions have not been introduced. The recreational component of these

fisheries varies from over 90% in some areas of New Zealand and nearly 50% in South Africa, to as low as 3% in Tasmania.

In the Perth Roe's abalone fishery, where fishers can easily access a substantial portion of the abalone population, the recreational catch is likely to be a particularly important component of the total catch. Because Perth is also the focus of the commercial fishery, providing approximately one-third of the annual commercial catch for the state, understanding the total catch in the Perth fishery is an important component of managing the resource.

The total catch in the Perth area needs to be determined on a scale that is appropriate to the stock structure for the species. The genetic neighborhood of populations in the Perth area has been found to be less than 13 km (Hancock 2000). Therefore, catch and effort information for this intensively harvested species should be considered in units of less than 13 km, or units of semi continuous reef. The finest spatial resolution of the commercial catch records during the period of this study was 10 nm sections of coast. These sections reasonably approximate units of semi continuous reef.

The objective of this study is to: (1) estimate the recreational catch and effort for each 10 nm section of the Perth Roe's abalone



fishery and (2) compare the relative catches of the recreational and commercial sectors over the study period.

This study has been complemented by the development of an independent telephone survey designed to estimate the recreational abalone catches from the whole Western Australian coast. The survey was stratified to provide more detail for the Perth fishery (Hancock et al. 2003). Catch estimates from the Perth area derived from telephone and creel surveys provide a rare opportunity to compare independent recreational catch estimates.

## MATERIALS AND METHODS

A methodology for estimating the recreational catch and effort was developed using an intensive examination of data obtained between 1997 and 1999. This technique was then used to obtain estimates for subsequent years as an on-going monitoring program. The development of the estimation procedure is described in detail and results obtained by applying the described technique to data collected from 2000 to 2003 are reported.

### *Spatial Stratification of the Perth Recreational Fishery*

The Perth recreational abalone fishing area has been stratified into zones of high and low usage, based on extensive observations (Fig. 1). The highest levels of utilization are in areas of reef adjoining the mainland shore. Within this range the areas most targeted by recreational fishers have been identified (Fig. 2). The low usage zone is the remainder of the area between Cape Bouvard and Wedge Island (Fig. 1).

### *Environmental Conditions*

For each day of the fishing season the prevailing fishing conditions are likely to influence the distribution of effort and the catch rate of recreational fishers. To quantify fishing condition and enable fishing conditions to be accounted for when estimating effort, a 10-point index was developed, with very good conditions equal to 0 and very poor conditions equal to 9 (Table 1). The fishery operates on the reef platform or shallow subtidal areas of an exposed coastline. Wave and tide height are thus especially important, with minor influence from light level, as determined by cloud and rain. Wave heights were obtained from the Department of Transport "Deep Channel" wave station in section 39 (31°58'40"S 115°41'12"E, Fig. 1). Recordings were divided into wind-driven wave and swell height, based on wave period. On mornings with a dominant easterly (offshore) wind, swell height was used, and on mornings with a dominant westerly (onshore) wind, the sum of swell and wind-wave height was used. Tide heights were obtained from the Department of Transport, Fremantle recording station. Cloud cover and rainfall were obtained from the Bureau of Meteorology, Mt. Lawley station.

### *Effort—High Usage Zone*

Within the high usage zone there were 20 discrete areas from which counts of the number of fishers were taken (Fig. 2). All fishers were visible from raised vantage points within each area. The length of coast adjacent to the 20 areas ranged from 130 m to 895 m, giving a total of 6,924 m of coast in the areas counted, or 60% of the total length of reef which provides suitable habitat for Roe's abalone (11,553 m) in the high usage zone (Fig. 2). Count areas were located systematically throughout each 10-nm section to achieve an approximately even geographical spread of sampling effort, whereas the exact size and position of each area was ad-

justed so that unambiguous start and end points could be described from land and air, using purpose-drawn maps. Count areas represented reefs of high and low accessibility, and greater and lesser usage by fishers.

Department of Fisheries research staff and volunteer fisheries liaison officers (VFLOs) conducted instantaneous counts of the numbers of fishers. VFLOs were instructed to take a minimum of one count of fishers actively engaged in fishing from their area at 7:10 AM (710 count), and encouraged to take 3 or more counts throughout the fishing period. Research staff conducted a count every 10 min from 7:10 AM to 8:20 AM, a total of eight counts for the 1.5-h period. Effort was calculated in fisher minutes for each count area using the research samples and the VFLO samples for which there were three or more instantaneous counts. The VFLO samples were used only if a 710 count was taken, and at least one count was taken from each half hour period between 7:30 to 8:00 AM and 8:00 to 8:30 AM. The calculation of effort can be illustrated by plotting the histogram of instantaneous counts taken during the fishing period at a site, against the time the counts were taken (Fig. 3). The area of the histogram describes the effort in fisher minutes. For the volunteer samples, the area of each bar of the histogram or effort described by each count was determined by the timing of each instantaneous count. This can be described as in the following equation:

$$E = C_1 \left( (t_1) + \frac{(t_2 - t_1)}{2} \right) + \sum_{i=2}^{f-2} C_i \left( \frac{(t_i - t_{(i-1)})}{2} + \frac{(t_{(i+1)} - t_i)}{2} \right) + C_f \left( \frac{(t_f - t_{(f-1)})}{2} + (90 - t_f) \right)$$

where  $E$  is the effort for the count area on a day in fisher minutes,  $C$  is an instantaneous count of fishers,  $t$  is the time the instantaneous count was taken in minutes from the start of fishing at 7:00 AM,  $i$  indicates the count number for intermediate counts between 1 and  $f$ , the final count for the fishing period.

Analysis of fisher participation was initially undertaken using data collected during the 3 years from 1997 to 1999. Counts of fisher participation were taken from a possible total of 20 areas per fishing day, for each of the six fishing days in 1997, 1998 and 1999 (Table 2). There were two kinds of measures of effort during 1997 to 1999, those calculated from the research samples with eight counts per day (41 cases), and those calculated from VFLO samples with three to five counts per day (146 cases). Each type of sample included a 710 count. Table 2 also contains two types of missing values where there were insufficient data to calculate effort for an area on a given day: those for which the available effort information was only one 710 count (58 cases), scenario a; and those for which there was no effort information at all (115 cases), scenario b. The missing values were estimated using two predictive generalized linear models that were developed with the information contained in the 187 research and VFLO samples. The use of volunteer staff limits the ability to prescribe completely the timing and number of counts taken during a fishing day and the areas counted per day, resulting in missing data for some areas.

For the first model, scenario a, the research and VFLO sampling was used to establish a relationship between the 710 count and fishing effort at an area for a given day, after accounting for variables and interactions that may affect this relationship. This model then used the 710 count to predict the missing effort estimate for the 1.5-h fishing period (Table 3). The appropriate predictive model to estimate the effort for scenario b was determined

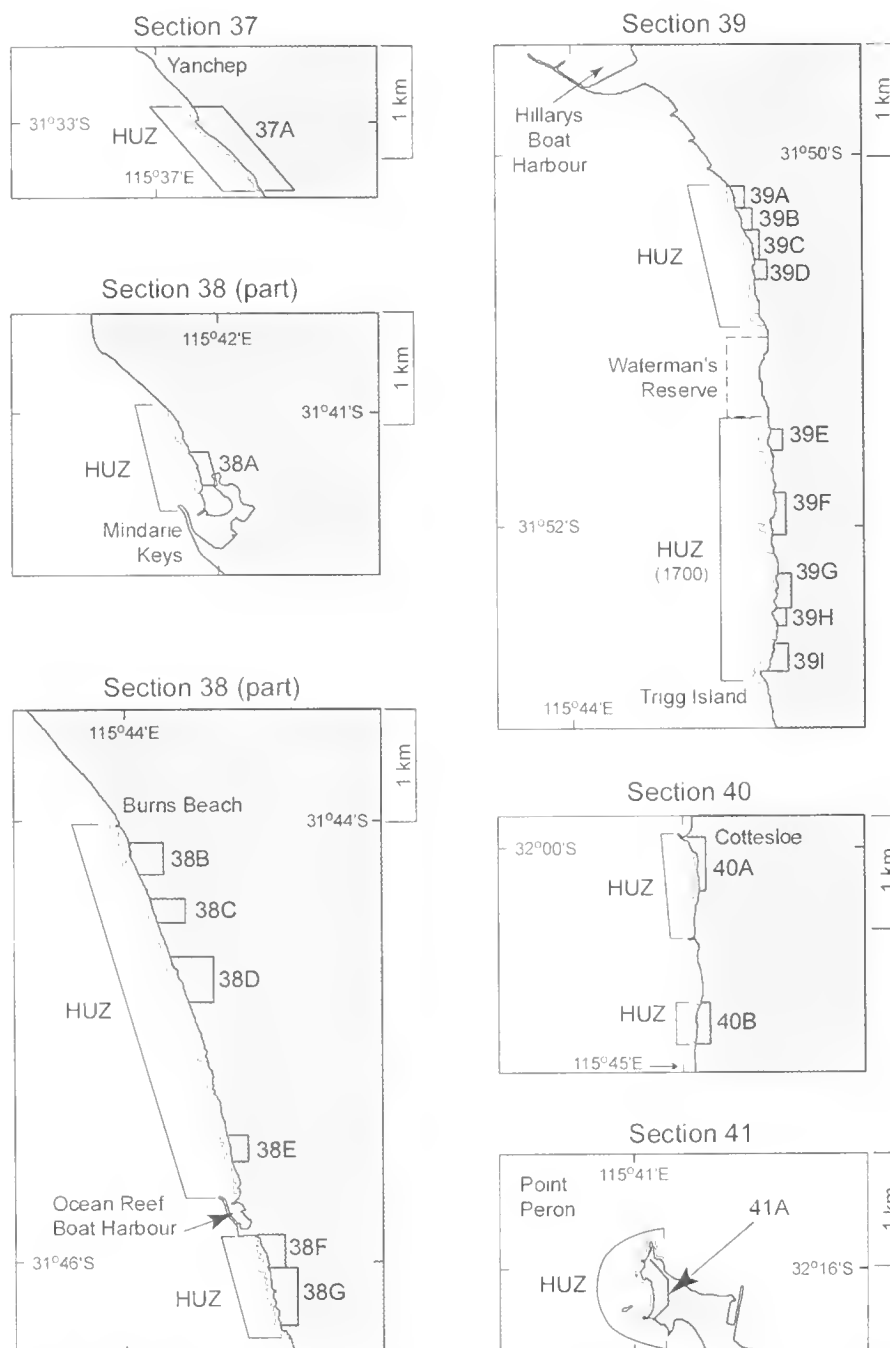


Figure 2. Maps of the high usage zones (HUZ) of each 10 nm section of the Perth abalone fishery, indicating the 20 areas where fishers were counted for estimation of effort.

by examining the relationship between the effort calculated from research and VFLO sampling and the variables that most influence effort. The variables investigated were data source (research or VFLO), year, day, count area, and fishing condition (Table 4). As a result of increasing variances in effort with increasing 710 count levels, effort and 710 count values were log transformed. Because geometric means are calculated from log transformed data the predicted values of effort will be biased. A correction for this bias was included by multiplying all back transformed estimates by  $10^{(S^2/2)}$  where  $S^2$  is the residual mean square error for the model used to make the estimate (Baskerville 1972, Caputi et al. 1979).

The models developed from the 1997 to 1999 data were applied

to the counts of fisher participation for 2000 to 2003. During this period there were no research counts taken every 10 min from the same area. There were 120 possible count area/day combinations per year. For each combination there is either a minimum of three counts including the 710 count, a 710 count only or no count information (Table 2).

Effort for the individual count areas was summed for each 10 nm section and then extrapolated to produce an estimate of daily effort for the whole 10 nm section. The scaling factor used was the ratio of the total number of fishers in the high usage zone, to the number of fishers in the 20 count areas. This ratio was obtained from analysis of digital video taken from light aircraft at 150 m

TABLE 1.

Environmental measures included in an index of fishing conditions for the Perth recreational Roe's abalone fishery. Category values are summed across measures (columns), by choosing the corresponding value for the category from each column to give an overall index on a scale of 0 to 9. Wave height is the mean of two recordings at 7:00 and 8:00 AM, tide height is the mean of four half-hourly recordings taken from 7:00 AM, cloud cover refers to the mean of two observations at 7:00 and 8:00 AM, and rainfall refers to the period from 6:00 to 9:00 AM. Category values for the four environmental variables are summed to derive the fishing condition index.

Category Value	Wave Height (m)	Tide Height (cm)	Cloud Cover	Rainfall
0	0.00–0.30	to 30	0%–25%	Nil
1	0.31–0.60	31–60	25%–50%	Rain
2	0.61–0.90	61–90	—	—
3	0.91–1.20	>90	—	—
4	>1.20	—	—	—

(500 feet). Digital video was first successfully used on day 5 of the 2000 season. Previous attempts to use analog video footage to obtain this ratio were unsuccessful because of inadequate resolution. The ratio obtained for 2000 was applied to 1997 to 1999. A separate ratio was obtained from digital video taken on one day of each subsequent year, and used to extrapolate effort estimates for that year (Table 5).

#### Effort—Low Usage Zone

In the low usage zone information on effort was collected from aerial counts of fishers, with no land-based surveys. Aerial surveys were conducted on two days of each fishing season between 1997 and 2002, days 2 and 5 in 1997 and 1999 to 2002, days 1 and 4 in 1998, and on one day (day 2 only) for 2003. Counts were taken from 500 feet, beginning at Cape Bouvard in the south between 7:00 AM and 7:30 AM, and ending at Wedge Island in the north between 8:00 AM and 8:30 AM. Fishers on each reef were few enough to allow direct counts of abalone fishers. Direct aerial counts were not possible for the high usage zone because of the high density of fishers.

Aerial counts were assigned to the same 10- or 15-min time intervals used to calculate effort from the research counts, and an effort value in fisher minutes for that interval was calculated. To estimate effort for the 1.5-h period from this one count it was assumed that the distribution of fishing effort during the fishing period in these areas was the same as in the high usage zone, where research counts were taken. Thus,

$$E_L = \sum_{i=1}^n C_i T / P$$

where  $E_L$  is the effort for the low usage zone portion of a 10-nm section,  $n$  is the number of reef counts of fishers in a section,  $C_i$  is an aerial count of fishers on reef  $i$ ,  $T$  is the 10 or 15 min period the count is allocated to and  $P$  is the mean proportion of the total effort for the research areas attributed to that 10 or 15-min period.

To estimate the effort for the low usage zone on days when aerial surveys were not conducted, the effort in each 10-nm section of this zone was assumed to be a constant proportion of the effort

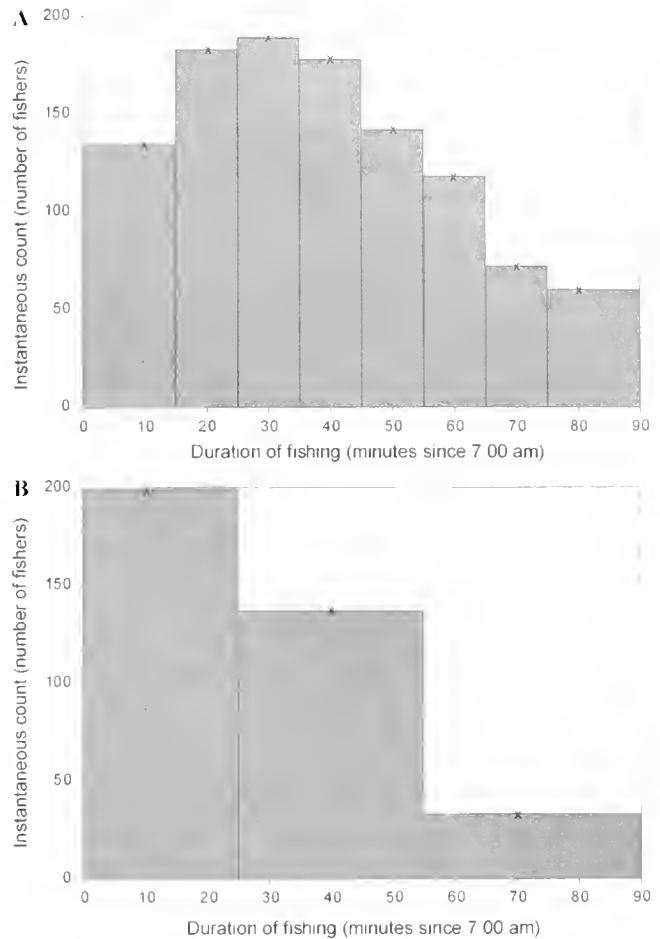


Figure 3. Example histograms to illustrate the calculation of effort from (A) the eight counts of a research sample, and (B) three VFLO counts as a worst case example of a VFLO sample where three to five counts were made. X indicates the position of each instantaneous count of the number of fishers.

in the count areas of the high usage zone for each year, based on the two days with aerial survey information each year.

#### Catch Rate and Catch

Catch rates were obtained from interviews of fishers as they returned to the beach after fishing. Interviews were conducted by VFLOs, who recorded the time of the interview and the number of

TABLE 2.  
Number of effort counts of different types for 1997–2003.

Year	Research Counts 8 per Day	Volunteer Counts 3–5 per Day	Count at 7:10 AM Only	Number of Missing Counts
1997	17	33	27	43
1998	12	52	24	32
1999	12	61	7	40
2000		44	14	62
2001		73	5	42
2002		65	6	49
2003		39	12	69

TABLE 3.

ANOVA model of the dependent variable log effort used to estimate effort for areas with only one 710 count (scenario a).

Source	df	Mean Square	F Ratio	P
log 710 count*	1	0.846	104.5	<0.001
Day*	1	0.185	22.8	<0.001
Area counted	19	0.015	1.9	0.024
Fishing condition*	1	0.041	5.0	0.026
log <sub>10</sub> 710 count × day	1	0.120	14.8	<0.001
log <sub>10</sub> 710 count × area counted	18	0.016	2.0	0.013
log <sub>10</sub> 710 count × fishing condition	1	0.090	11.1	0.001
Residual	144	0.008		

Asterisk indicates covariates.  $R^2 = 0.951$ .

abalone taken, when the fisher commenced fishing, and how long they had been fishing. Where two or more fishers were pooling their catch, the number of abalone was divided equally between fishers. Interviews were not restricted to the 20 areas with counts, but were conducted throughout the high usage zone, and from the Mandurah area, at the southern extremity of the Perth fishery. Variations in catch rates, calculated in abalone per fisher hour, were examined using ANOVA for the years 1997 to 1999. Factors examined as possible influences on catch rate were 10 nm section, year, day and fishing condition. Catch rates were skewed toward high values and were normalized using a log (catch rate + 0.05) transformation.

Multiplying the estimates of effort and mean catch rate per day provided an estimate of catch for the recreational sector, expressed as a number of abalone.

The commercial catch in the Perth fishery is monitored in whole weight only, so the mean weight of recreationally caught abalone was investigated to allow conversion of the recreational catch to whole weight for comparison with commercial catches. Weigh stations were established at an access point, the catch of fishers leaving the beach was weighed and the number of abalone per catch recorded, giving mean abalone weight per catch. During the 1999 season catch weights were recorded from 1 site per day on days 2, 4 and 6 of the fishery. During 2000 weight measurements were taken from between 3 and 7 sites per day, from a total of 10 sites, or 26 site-day combinations, including the 3 sites sampled in 1999. The relationship of mean abalone weight and

TABLE 4.

ANOVA model of log effort, used to estimate effort for areas with no 170 count, for areas with at least one instantaneous count taken each year (scenario b).

Source	df	Mean Square	F Ratio	P
Year	2	0.260	19.6	<0.001
Day	4	0.033	2.5	0.045
Area counted	19	0.771	58.1	<0.001
Fishing condition*	1	0.023	1.7	0.191
Day × fishing condition	4	0.081	6.1	<0.001
Area counted × year	28	0.031	2.3	<0.001
Residual	127	0.013		

Asterisk indicates covariate.  $R^2 = 0.929$ .

TABLE 5.

Percentage of the total number of fishers that were within the count areas used to estimate effort for the high usage zone of each section of the Perth recreational Roe's abalone fishery.

Section	% in Count Areas			
	2000, Day 5	2001, Day 2	2002, Day 2	2003, Day 2
37	100.0	100.0	100.0	100.0
38	62.9	64.9	67.1	63.6
39	90.4	93.3	86.8	94.7
40	75.0	70.0	100.0	Closed
41	75.0	100.0	87.5	64.3

site, day and fishing condition was examined using ANOVA of data collected during the 2000 season. The relationship between mean abalone weight and site, year and fishing condition was examined using ANOVA of the data collected from the three sites sampled during the 1999 and 2000 seasons.

#### Commercial Sector

Commercial catch is reported for each 10 nautical mile section of the Western Australian coast from Shark Bay to the South Australian boarder. Catches in whole weight are reported on a daily basis as part of the quota management.

## RESULTS

#### Effort—High Usage Zone

Linear regression between log effort and log 710 count, for the 187 research and VFLO samples combined, for 1997 to 1999 data, indicated a strong positive relationship ( $R^2 = 0.854$ ,  $P < 0.001$ ). For scenario a, where a 710 count is available, an analysis of variance with log effort as the dependent variable, was used to consider the effect of the data source (research or VFLO samples) together with log 710 count, and other variables year, day, count area and fishing condition. The data source was significant ( $P = 0.028$ ), but accounted for only 0.2% of the sum of squares after accounting for the effect of the other variables. This is a minor influence on the model where the main effects account for 92.3% of the total sum of squares. There was no significant first order interaction between the data source and the other five explanatory variables. Therefore, the research and volunteer samples were pooled for further analysis. The main effect of year was not significant ( $P = 0.554$ ) and there was no interaction between year and log 710 count ( $P = 0.142$ ). Therefore, year was dropped from further analyses. Day and count area were significant as main effects ( $P < 0.001$ ). Fishing condition proved to have important interactions and was retained. The ANOVA was repeated after removing data source and year from the model. The main effects and the most influential first order interactions are shown in Table 3. For this model the interactions of log 710 count with day, area and fishing condition account for 1.4%, 1.3% and 0.4% of the total sum of squares respectively. This model (Table 3) was used to estimate missing effort values for areas with only the 710 count. The effect of day was positive and significant, indicating that as the season progressed, and abalone were progressively fished down, the effort required for a given number of fishers to obtain the daily catch increased. The effect of fishing condition was also

positive and significant, indicating that as fishing conditions deteriorated the average individual fisher spent more time on the reef searching for their catch. This is because abalones tend to occupy the area where waves break, on the seaward section of the reef platform, and it becomes more difficult to fish as the waves become larger. This difficulty is compounded by an increase in the depth of water over the reef platform (tide height), because the majority of fishers wade without mask, snorkel or weights.

For scenario b, the ANOVA model of the five likely influences on log effort without a 710 count showed that data source was not significant as a main effect ( $P = 0.051$ ) and accounted for only 0.3% of the total sum of squares. There were no significant first-order interactions between data source and the other explanatory variables. Therefore, research and volunteer samples were pooled. All other variables were significant as main effects ( $P < 0.001$ ). For the remaining variables (year, day, count area and fishing condition) the dominant first-order interactions are year  $\times$  count area and fishing condition  $\times$  day (Table 4). These interactions account for 3.7% and 1.5% of the total sum of squares, respectively. This model (Table 4) was used to estimate the effort for areas with no counts for a given day. For this model effort increased as the season progressed, indicating that fishers continued to participate despite declining numbers of legal-sized abalone later in the season, and the time spent obtaining the catch increased after other factors were accounted for. The relationship with fishing condition was negative, indicating that on average as fishing conditions deteriorated there were fewer people fishing for the day, although the average fisher that did participate fished for longer to obtain their catch (scenario a).

Inclusion of year in an interaction term in the model means that it is not possible to estimate effort for a count area for any year in which there were no counts on any day during that year. For these area-year combinations, effort has been estimated using the main effects only, which still explain 87.7% of the variation. The same relationship was used to estimate the effort values for the 11 area-year combinations with no count information for 2000 to 2003.

Extrapolation of effort from the count areas per section, to the total for the high usage zone of each section, was done using the ratio of the total number of fishers, to the number within the count areas for each section. This ratio was first available from day five of the 2000 season, and was applied to the estimates for 1997 to 2000, the ratio used for extrapolation in subsequent years was available from sampling in that year (Table 5). Extrapolated estimates of effort over the total of the high usage reefs increased from 11,336 fisher-hours in 1997, to 19,216 fisher-hours in 2003 (Table 6), an increase of 70% over the 7 y.

#### *Effort—Low Usage Zone*

The mean percent of the effort from the research areas, that was attributed to each 10 or 15 min count period, ranged between 22.1% for the 710 count to 6.1% for the count at 8:20 AM (Table 7). The relationship was used to estimate the proportion of the effort for a fishing day that was described by each instantaneous count in the low usage zone. The estimates of effort indicate negligible fishing activity in sections 33, 34, 35 and 42. Between 1997 and 1999 the effort in the low usage zone decreased slightly (888–565 h), before beginning a rapid, steady rise to 1910 h in 2003. The increase in effort in the low usage zone has been most notable in sections 41 and 43 toward the southern extent of the fishery.

The total effort for the high and low usage zones combined is

dominated by section 38, which received between 43% (2000) and 55% (1998) of the total effort during the study period, averaging 49%. Section 39 received between 32% (2003) and 44% (2000), averaging 38%. Sections 38 and 39 combined accounted for between 85% (2003) and 90% (1999) averaging 87%. The high usage zone accounted for an average of 94% of the effort during the 7-year study period (Table 6) varying from 91% (2003) to 97% (1999).

#### *Catch Rate and Catch*

Analysis of variance showed that year, day, section and fishing condition were significant main effects influencing catch rate between 1997 and 1999. Because of the large sample size (4,025) many of the factors and interactions are likely to be significant. Fishing condition accounted for 10.1% and day accounted for 3.1% of the total sum of squares. Section and year each accounted for less than 1% of the total sum of squares. When the most influential interaction terms are added to the model the fit to the data is slightly improved ( $R^2 = 0.190$ ), with fishing condition  $\times$  day accounting for 2.9% of the total sum of squares ( $P < 0.001$ ), and day  $\times$  section 1.4% of the total sum of squares ( $P < 0.001$ ). Given the minor influence of 10 nm section on the catch rate, and the large number of interviews, the respective daily mean catch rate was considered appropriate for estimating catch in number of abalone, on a daily basis, from the estimates of effort. The average catch rates varied between 16 and 32 abalone per hour for the days fished in 1997 to 2003.

A summary of the weight data collected between 1999 and 2003 is provided in Table 8. ANOVA results for the three sites sampled in 1999 and 2000 showed that fishing condition did not have a significant effect on mean weight. Site and year were both significant as main effects with no significant interaction between the two. The model of site and year as main effects explains 28.8% of the observed variation with 5.1% being because of site and 23.8% because of year.

Results for ANOVA of the 2000 weight data indicate that fishing condition is only significant in its interaction with site ( $P = 0.034$ ) when modeling mean abalone weight against site, fishing condition and the interaction of site  $\times$  fishing condition. In this case fishing condition accounts for only 1.2% of the total variation explained (26.7%) and is not considered important. When the model of day, site and day  $\times$  site is considered all terms are significant. The model explains 29.0% of the observed variation with 2.7% explained by the interaction of site and day, 2.7% explained by day alone and 23.6% explained by site. Examination of the mean abalone weight from all sites measured per day indicates the site  $\times$  day interaction is most strongly influenced by an unusually low mean weight from Cottesloe on day 6, a mean from a relatively small sample (9 catches), which, together with the small percentage of error explained, suggest that the interaction is not of great importance and that the mean weight of abalone is predominantly influenced by site.

To account for the few sites sampled in 1999, the ratio of the 2000 value for the 3 sites that were measured in both years, over the value for all sites measured in 2000, has been used to derive the value that would have been expected if all sites were measured during the 1999 season. These seasonal means have been used to convert catch in number of abalone to catch in kilograms whole weight for 1999 to 2003 (Table 9). As no mean weight information is available for 1997 and 1998 the average of 1999 and 2000

TABLE 6.

Estimates of effort (fisher hours) for the low (LUZ) and high (HUZ) usage zone portion of each 10-nm section of the Perth recreational abalone fishery. Effort is from the aerial survey counts on the days indicated. LUZ % is the mean of the aerial survey effort expressed as a percent of the total effort for the HUZ count areas for that day. LUZ effort is the sum of the two effort values derived from aerial surveys and the effort for the remaining 4 days estimated as a proportion (mean %) of the total effort for the HUZ count areas per day. HUZ is the total extrapolated HUZ effort per section, and Total is the sum of the LUZ and HUZ effort.

Year Section	1997			1998			1999		
	LUZ	HUZ	Total	LUZ	HUZ	Total	LUZ	HUZ	Total
33	32		32	0		0	0		0
34	0		0	67		67	14		14
35	66		66	0		0	0		0
36	152		152	129		129	120		120
37	175	254	429	188	523	712	55	605	660
38	40	6,115	6,155	60	8,498	8,558	107	8,189	8,296
39	0	4,311	4,311	0	5,187	5,187	0	6,243	6,243
40	37	483	520	0	504	504	49	404	453
41	165	172	337	45	173	218	98	245	343
42	55		55	7		7	6		6
43	166		166	123		123	117		117
Total	888	11,336	12,223	619	14,884	15,503	565	15,686	16,251

Year Section	2000			2001			2002			2003		
	LUZ	HUZ	Total	LUZ	HUZ	Total	LUZ	HUZ	Total	LUZ	HUZ	Total
33	14		14	0		0	0		0	134		134
34	10		10	0		0	21		21	0		0
35	0		0	0		0	84		84	0		0
36	126		126	63		63	146		146	313		313
37	107	723	830	7	1,037	1,044	130	667	797	213	1,047	1,260
38	115	6,693	6,808	25	7,930	7,955	131	8,316	8,447	162	11,008	11,170
39	0	7,042	7,042	0	7,392	7,392	0	7,400	7,400	0	6,804	6,804
40	41	316	357	37	192	229	64	61	124	77	0	77
41	240	165	405	701	118	819	584	90	674	535	357	892
42	27		27	20		20	45		45	18		18
43	198		198	204		204	391		391	459		459
Total	877	14,939	15,817	1,059	16,668	17,727	1,594	16,534	18,127	1,910	19,216	22,126

(104.6 g) has been assumed for the conversion of the recreational catch in number of abalone to whole weight for 1997 and 1998.

The low usage zone of the Perth recreational abalone fishery represents a minor portion of the total recreational catch (Table 9), averaging only 6.3%, of the total number of abalone taken from 1997 to 2003. Total recreational catches estimated in number of

abalone increased from 1997 to 2003 with a minor decrease in 2000 because of a decrease in the estimated catch from section 38 of over 50,000 abalone. The recreational catch is taken primarily from two sections, 38 and 39, which averaged 87.2% of the catch over the 7 years (85.0% to 89.5%). Catches from both sections 38 and 39 showed an upward trend over the 7-year period, with section 38 averaging 49.3% and section 39 averaging 37.9% of the

TABLE 7.

The mean percentage of effort per area counted per day, described by each time interval used to calculate daily effort for the research areas counted from 1997 to 1999.\*

Time Interval	Mean % Effort	±SE
7:00–7:15 AM	22.30	0.91
7:16–7:25 AM	16.10	0.44
7:26–7:35 AM	15.85	0.37
7:36–7:45 AM	13.62	0.28
7:46–7:55 AM	11.09	0.33
7:56–8:05 AM	8.54	0.40
8:06–8:15 AM	6.31	0.37
8:16–8:30 AM	6.17	0.54

\* Standard error,  $n = 41$  (R in Table 3.2).

TABLE 8.

Summary of weigh station data collected from the Perth Recreational Roe's abalone fishery 1999 to 2003.

Year	Areas Surveyed	Day/Area Combinations	Catches Weighed	Number of Abalone	Mean Weight/ Abalone
					(Weighted, g)
1999	3	3	133	2,320	*119.4
2000	10	26	762	14,516	89.8
2001	14	47	1,854	35,838	91.6
2002	11	36	1,702	31,909	89.7
2003	9	29	1,563	29,760	96.4

\* Weighted mean from 3 sites sampled, adjusted by the ratio of the mean weight for those 3 sites to the mean from the 10 sites using the 2000 data.

TABLE 9.

Estimates of recreational catch (kg) in the high (HUZ) and low (LUZ) usage zones of each 10 nm section, with combined recreational catch (Rec.) commercial catch (Com.) and the total catch from the Perth abalone fishery, 1997–2003. Blanks indicate no HUZ, or that these sections lie beyond the northern boundary of the Perth fishery for the commercial sector (sections 33 to 35). Catch weights for 1997 and 1998 have been derived using a mean weight of 104.6 g obtained for 1999–2000.

Year Section	1997					1998				
	HUZ	LUZ	Rec.	Com.	Total	HUZ	LUZ	Rec.	Com.	Total
33		84	84		84		0	0		0
34		0	0		0		166	166		166
35		178	178		178		0	0		0
36		415	415	436	851		319	319	188	507
37	702	475	1,177	354	1,531	1,334	467	1,800	0	1,800
38	16,698	108	16,806	14,184	30,990	20,973	147	21,121	11,562	32,683
39	11,662	0	11,662	9,925	21,587	12,812	0	12,812	5,878	18,690
40 Is.	1,342	101	1,443	207	1,650	1,235	0	1,235	0	1,235
41	454	454	908	11,045	11,953	440	111	551	6,563	7,114
42		150	150	0	150		17	17	0	17
43		455	455	0	455		304	304	0	304
Total	30,858	2,421	33,279	36,151	69,430	36,794	1,532	38,326	24,190	62,516

Year Section	1999					2000				
	HUZ	LUZ	Rec.	Com.	Total	HUZ	LUZ	Rec.	Com.	Total
33		0	0		0		26	26		26
34		40	40		40		19	19		19
35		0	0		0		0	0		0
36		341	341	17	358		236	236	363	599
37	1,636	159	1,795	269	2,064	1,362	201	1,563	641	2,204
38	22,934	301	23,235	12,242	35,477	12,573	217	12,790	10,516	23,305
39	17,282	0	17,282	9,259	26,541	13,178	0	13,178	5,075	18,254
40 Is.	1,112	136	248	153	289	588	77	665	0	665
41	685	276	961	13,924	14,885	309	451	760	19,718	20,478
42		16	16	0	16		52	52	0	52
43		337	337	228	565		371	371	197	568
Total	43,649	1,607	45,256	36,091	81,347	28,010	1,649	29,660	36,509	66,169

Year Section	2001					2002				
	HUZ	LUZ	Rec.	Com.	Total	HUZ	LUZ	Rec.	Com.	Total
33		0	0		0		0	0		0
34		0	0		0		40	40		40
35		0	0		0		160	160		160
36		158	158	0	158		280	280		280
37	2,625	18	2,644	258	2,902	1,254	258	1,512	491	2,003
38	19,739	63	19,802	15,430	35,232	16,768	258	17,026	16,038	33,064
39	18,342	0	18,342	6,232	24,574	14,550	0	14,550	4,305	18,855
40 Is.	478	93	571	210	781	125	125	249	761	1,010
41	292	1,745	2,037	13,277	15,314	177	1,159	1,337	14,370	15,707
42		51	51		51		86	86		86
43		507	507		507		774	774		774
Total	41,476	2,636	44,112	35,406	79,518	32,875	3,141	36,016	35,965	71,981

Year Section	2003				
	HUZ	LUZ	Rec.	Com.	Total
33		320	320		320
34		0	0		0
35		0	0		0
36		749	749	74	823
37	2,521	511	3,032	266	3,298
38	26,262	387	26,649	15,668	42,317
39	16,347	0	16,347	6,054	22,401
40 Is.	0	185	185	1,198	1,383
41	860	1,280	2,140	12,601	14,741
42		42	42		42
43		1,099	1,099	145	1,244
Total	45,990	4,574	50,564	36,007	86,571

total recreational catch from the Perth fishery measured as the number of abalone taken.

The effect of the variation in mean abalone weight, on the catch estimates in whole weight, is to accentuate increases in catch between 1997 and 1999 and to accentuate decreases between 1999 and 2000 (Table 9). The total recreational catch estimate increased by 36.0% between 1997 and 1999, then decreased by 34.5% between 1999 and 2000. This was driven by an increase in catch of 39.1% for section 38 and 48.0% for section 39 between 1997 and 1999, and a subsequent decrease in catch of 45.0% for section 38 and 23.8% for section 39 between 1999 and 2000. The total recreational catch increased by 51.9% between 1997 and 2003.

The telephone survey that began in 1999 (Hancock et al. 2003) provides an independent estimate of the recreational Roe's abalone catches from the Perth fishery as a whole. These estimates compare favorably with the results of the present field survey, with field survey results falling within the 95% confidence intervals of the telephone survey estimates (Fig. 4). The independent estimates corroborate the increasing trend in the recreational catch of Roe's abalone from the Perth fishery.

#### Combined Commercial and Recreational Catch

The commercial sector has caught its quota from the Perth fishery in recent years, the majority of the catch (97% to 99%) coming from the 10 nm sections 38, 39 and 41 during this study (Table 9). Catches from sections 38, 39 and 41 averaged 40%, 20% and 38% of the total commercial catch from the Perth fishery over the seven years. Nearly all of the commercial catch from section 41 comes from the low usage portion, an area of very low recreational catch.

The commercial catches are atypical for 1998 because of a one-off reduction of the commercial quota to 24 t for the 6-month period from October 1998 to March 1999 inclusive. The commercial catch from section 38 increased during 2001 to 2003, whereas the catch from section 39 decreased during 2000 to 2003, with any difference being made up from section 41.

The commercial and recreational catches are not comparable for 1998 because of the shortened commercial season. Combined commercial and recreational catches have increased 25% between

1997 and 2003, going from 69.5 to 86.6 t respectively (Table 9). The recreational catch from the Perth fishery has increased from 47.9% to 58.4% of the combined recreational and commercial catch between 1997 and 2003, although the proportion has varied during that period. Section 38 is the most productive of the overall fishery, producing an average of 45% of the annual catch, with section 39 producing an average of 29% and section 41 producing an average of 18%.

#### DISCUSSION

The cost of survey personnel has commonly been a major limitation on the design of creel surveys to estimate catch and effort in recreational fisheries (e.g., Jones et al. 1990, Newman et al. 1997). In this survey the use of numerous volunteers has largely overcome this limitation. The use of volunteers introduces other limitations, the primary one being a reduced ability to prescribe the areas surveyed on a daily basis. This limitation has been addressed largely by the use of predictive GLM models to estimate the missing effort values. The use of these estimation techniques was made practical by the highly predictable patterns of recreational fishing effort within the high usage zone areas counted, with the 710 count proving to be an effective indicator of effort ( $R^2 = 0.951$ , Table 3). The estimation of effort in areas for which no counts were available was also achieved with a high degree of confidence, with  $R^2$  values of 0.929 and 0.877 for the two models used to estimate missing effort values (Table 4).

Creel surveys have been conducted previously using roving or access point methods, requiring stratification of the fishery by time and space (Malvestuto & Knight 1991). The structure of the Perth abalone fishery makes it possible to sample the entire fishing area of the low usage zone for one third of the fishing days, requiring the assumption that the percent of effort observed during this period in the low usage zone is representative of the remaining 4 of the 6 fishing days. It is possible to obtain measures of effort from the whole fishing period for the high usage zone, leaving space as the dimension requiring stratification. This study uses direct observation of effort from 60% of the high usage zone. Extrapolating effort from count areas to total high usage zone per section, using the ratio of the total number of fishers in the high usage zone, to the number within the combined count areas from only 1 day assumes that the ratio for that day is representative of all fishing days considered. This assumption deserves further examination in the future.

The method of estimating effort also assumes that all individuals on the reef were participating in the abalone fishery. Effort may be over estimated if there were a significant number of people that were present on the reef that were not intending to collect abalone, and subsequently not interviewed later. Evidence that this was not the case is that very few people refused an interview, and that on each day there were interviews of people that had caught zero abalone. Because Roe's abalone tend to occupy the surf zone, it is unlikely that large numbers of people would venture into this environment without reason. The occasional angler present on the reef was easily identified, and not included in the count of abalone fishers.

An advantage of this method of catch estimation, over telephone or mail surveys, is that the estimate takes into account catches by unlicensed fishers on the reef, or by fishers who have returned to fish a second quota for the day. This component of illegal fishing is captured by the direct observation of effort. Much

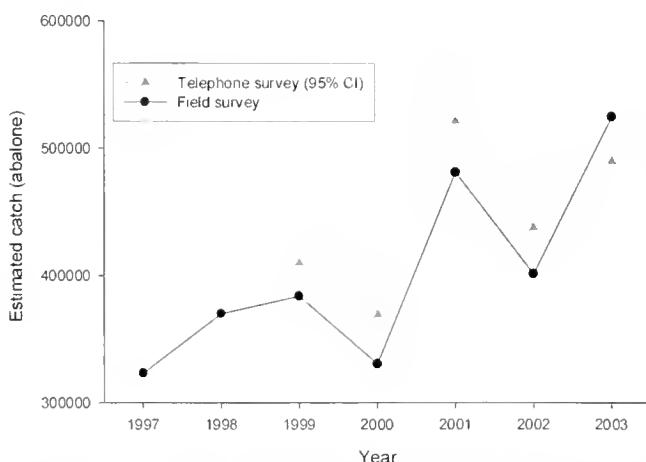


Figure 4. Comparison of recreational catch estimates in number of abalone from the field surveys and the telephone surveys ( $\pm 95\%$  confidence intervals) for the Perth recreational Roe's abalone fishery. Phone survey data provided by T. Baharthah and N. Sumner (unpublished data).



of the illegal take of more than the prescribed bag limit is also accounted for by using direct observations of catches. Other illegal activity such as out of season poaching remains unaccounted for in this study.

The low usage zone contributed between 3.5% and 9.0% of the recreational effort, and aerial surveys provide a cost effective technique for estimating this proportion. For the low usage zone, conversion of instantaneous counts to effort assumes that the distribution of fishing effort throughout the fishing period in this zone is similar to the average distribution of effort in the research areas counted in the high usage zone. Fishing is allowed throughout the fishing day in areas more than 800 m offshore. Although the amount of fishing that is conducted outside the core fishing time in offshore areas of the low usage zone is known to be low, this provides scope for an underestimation of effort. Areas affected are the low usage reefs of section 40, 42 and section 41 for 1997 only. The offshore areas of section 41 were brought under the mainland regulations in 1998. This change did not produce any discernible impact on the estimates of effort for this area, so any effects on estimates of total effort are likely to be minor.

Within the recreational sector the fishing competence and the confidence of individuals in the water vary considerably. This is reflected in the range of individual catch rates on any day, with some fishers returning to the beach with few abalone for all but the best fishing conditions and some fishers taking their bag limit of 20 abalone in as little as 5 min. It is therefore necessary to calculate mean catch rates from a large number of interviews. For this study catch rates have been calculated from between 98 and 414 interviews per day. Catch rates for the Perth recreational Roe's abalone fishery are high compared with other abalone fisheries (e.g., Lyle & Smith 1998, Worthington et al. 1998), and fishing times are low, reflecting the high densities of this species.

The recreational catches have been converted to estimates of whole weight for comparison with commercial catches. The consistent and highly significant change in the mean weight of abalone taken in the recreational fishery between 1999 and 2000 highlights the possibility that assuming a mean weight for the 1997 and 1998 seasons, on the basis of measurements made in 1999 and 2000, may introduce an appreciable bias in the estimation of catch weight for these years. Trends in catch indicated by number of abalone taken would be more reliable for these years, and comparisons with the commercial catch for 1997 and 1998 should be interpreted with added caution. The mean weights for 2000 to 2003, which have been sampled from at least 9 sites have been relatively consistent varying from 90 to 96 g. The high mean weight in 1999 may have been affected by the low level of sampling in the first year when mean weights were sampled.

The recreational catch from the high usage zone is highly dominated by sections 38 and 39, and there was a substantial decrease in fishing effort in 2000 (Table 6), particularly evident in section 38. Reasons for this decrease in effort are likely to include fishing conditions that were more challenging.

The nature of recreational catch estimates means that they are generally based on a set of assumptions that are difficult to test. This led Pollock et al. (1994) to conclude that the only real test of the accuracy of these estimates is corroboration from two or more independent estimates. Given the time and cost involved in estimating recreational catches, the availability of two independent estimates is unusual. The telephone survey was used to estimate recreational abalone participation rates, fishing effort and catch for the three exploited species of abalone in Western Australia. A

stratified random sample comprising about 800 of the up to 21,000 abalone license holders were contacted by phone (Hancock et al. 2003). The close agreement between the two independent estimates of the recreational Roe's abalone catch (Fig. 4) greatly increases the confidence that each represents a valid estimate of the catch for this fishery.

In no other abalone fishery in the world is there such a focus of fishing activity adjoining the suburban area of a major city. Also, most abalone species throughout the world are predominantly subtidal and occur in lower densities, meaning that fishers require a higher level of expertise and equipment to access them. The more diffuse nature of most abalone fisheries tends to favor mail or telephone surveys as a means of estimating the recreational catch and effort (Weithman 1991) where recreational fishing license information can be used to target fishers.

Abalone fisheries throughout the world are exploited to different extents by the commercial and recreational sectors, but the recreational catch, where it has been estimated, is generally an important component of the legal catch, with recreational fishing effort tending to increase (Teirney et al. 1997, Anon. 1998, 1999, Bradford 1998, Lyle & Smith 1998, Worthington et al. 1998). The combination of their high value and accessibility makes it important to monitor catch trends in these fisheries.

In the Perth fishery the Roe's abalone catch is high, reaching 42t for a 10 nm section of coast, and concentrated in just 30 nm (sections 38, 39 and 41). Of these, section 41 is nearly exclusively fished by the commercial sector. Sections 38 and 39 are the most heavily exploited, with the recreational sector taking between 54% and 72% of the catch from these sections during the 7-year period of this study. Examination of the population structure of Roe's abalone has demonstrated that individual stocks are separated by less than 13 km of coast (Hancock 2000). This scale is well represented by the patches of semicontinuous reef within each 10 nm section of the Perth fishery. The present results provide catch and effort information on a scale of the individual biological stocks within the fishery. Any stock assessment for this fishery should be conducted on the same spatial scale.

The recreational catch increased 65% during the period of the study, and now exceeds the commercial catch, whereas the commercial catch is fixed via quotas. This study provides the catch and effort information required to manage this intensively used fishery on the scale of the biological stocks that make up the fishery. This has been achieved despite the fine scale spatial subdivision of individual stocks.

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## TRANSPORT AND RECRUITMENT OF SILVER-LIP PEARL OYSTER LARVAE ON AUSTRALIA'S NORTH WEST SHELF

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**ABSTRACT** Silver-lip pearl oyster (*Pinctada maxima*) spat surveyed in the Eighty Mile Beach section of the North West Shelf have been used in conjunction with outputs from a particle dispersion model to identify likely spawning grounds. The dispersion model consisted of a 3-dimensional regional circulation model in which large numbers of individual particles were tracked over the period 1994 to 1999. From the settlement areas defined by the spat data, larvae were tracked back in time over their estimated pelagic phase of 24–31 days within the main spawning period of mid October to late December. The reverse calculation was also undertaken looking at larval dispersion from known broodstock populations. Results demonstrate that large tidal currents in the region move larvae back and forth across the shelf, whereas lower frequency currents influence their net transport. Whereas some model larvae traveled more than 60 km, most were transported less than 30 km. The model results suggest that spawning in the Eighty Mile Beach region is concentrated around the recently surveyed broodstock distribution between 8 and 15 m depth, with potential smaller contributions from the northeast. These spawning events are likely to lead to successful recruitment locally and alongshore to the southwest. They also feed larvae into neighboring shallow coastal environments (through tidal oscillations) and deeper waters to the west (~20 m). However, spat abundances seem to be low in these areas, suggesting that recruitment is strongly limited by habitat availability and possibly high mortality rates in shallow water. High local abundances of broodstock and spat observed occasionally in deeper water (~30 m) seem to be supported by intermittent larval transport from inshore populations. However, spawning in this area seems to contribute little to recruitment in the inshore populations.

**KEY WORDS:** *Pinctada maxima*, oysters, larvae, larval transport, recruitment

### INTRODUCTION

The pearling industry on Australia's North West Shelf (NWS) relies substantially on shell caught from the inshore waters of Eighty Mile Beach (Fig. 1). The most sought after species is the silver-lip pearl oyster, *Pinctada maxima* (Jameson) that produces the silver-white South Sea pearl. Oyster beds on the NWS provide "wild shell," which is collected by divers, before being seeded and placed back on the seabed in framed net enclosures or "panels." After recovering for a few months, they are transported to farms where the pearls can develop. These activities are managed as a gauntlet fishery, where oysters are caught at the optimum size for seeding (120–160 mm) leaving the larger mother-of-pearl (MOP, >175 mm) as breeding stock.

Recently surveyed MOP distributions show increasing abundance with water depth, whereas the abundance of recruits (120–160 mm) tends to decrease (Hart & Friedman 2004). This decoupling of broodstock and recruitment might be explained by net onshore transport of larvae spawned in deeper waters being offset by high rates of fishing and natural mortality in shallow waters. This is the basis for the long-standing belief within the pearling industry that deep "unfished" stocks could be broodstock source for the commercially fished inshore stock. However, prior to this study, such a hypothesis had not been tested.

It is clearly critical to the management of the fishery to understand the *P. maxima* reproductive cycle (Rose et al. 1990) and establish the geographical extent of the spawning stock supporting the fishery. The objective of this study is to use a particle dispersion model in combination with results from pearl oyster surveys to investigate larval transport and settlement. Specifically, poten-

tial settlement distributions have been estimated based on surveyed MOP spawning populations and compared with results from spat abundance surveys. The reverse calculation has also been made, estimating potential spawning distributions based on spat abundance data and comparing these with the MOP distribution data. This has allowed us to test the hypothesis that deep-water MOP stocks support inshore recruitment, and population connectivity relationships more generally, within *P. maxima* stocks.

### MATERIALS AND METHODS

Larval dispersion patterns were modeled by tracking particles transported by ocean currents estimated from a circulation model for the North West Shelf. Particle trajectories were used to estimate both potential settlement sites and potential spawning sites. Spawning distributions were based on a MOP survey conducted in September 2001, whereas settlement distributions were based on 3 years (2001 to 2003) of spat surveys. These surveys were focused off Eighty Mile Beach, where most of the fishery is concentrated.

#### Circulation Model

The circulation model was developed as part of the North West Shelf Joint Environmental Management Study (NWSJEMS 2002, Condie et al. 2003) and was based on code referred to as MECO (Model for Estuaries and Coastal Oceans). MECO is a general-purpose finite-difference hydrodynamic model applicable to scales ranging from estuaries to ocean basins. It uses a curvilinear orthogonal grid in the horizontal and fixed "z" coordinates in the vertical. A comprehensive description of the underlying theory is provided in the MECO Scientific Manual (Herzfeld et al. 2002). MECO has found previous applications in systems such as the Port Phillip Bay (Walker 1999), Bass Strait, the Great Australian Bight

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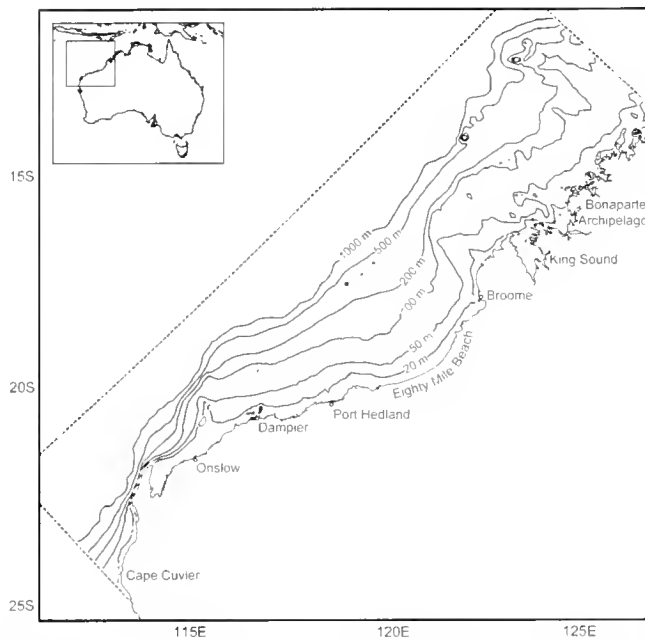


Figure 1. Map of the model grid region, bathymetry and key locations.

and Southeastern Australia (Bruce et al. 2001) and the Gulf of Carpentaria (Condie et al. 1999).

MECO was implemented for the NWS on a rotated latitude-longitude grid with horizontal resolution of approximately 10 km (Condie et al. 2005a). This grid extended from Cape Cuvier in the southwest to the Bonaparte Archipelago in the northeast and well beyond the shelf break (Fig. 1). The vertical resolution expanded from 3 m near the surface to a maximum of 200 m at its maximum depth of 1200 m. Truncating the depth at this level had little effect on the circulation, but significantly improved computational times. The bathymetry was prescribed by spatially averaging a 30 sec (0.9 km) product provided by Geosciences Australia onto the model grid.

Inputs required by the model included forcing caused by wind, atmospheric pressure gradients, and open boundary conditions such as temperature, salinity, and sea level. Wind fields were taken from the NCEP-NCAR 40-y reanalysis dataset (Kalnay et al. 1996). These fields had a 12 hourly time-step and a spatial resolution of  $1.8^\circ$ , which were linearly interpolated onto the model time-step and model grid. The interpolated product generally showed good agreement with locally measured winds at subdiurnal frequencies (Fig. 2a). However, smaller scale processes, such as the daily sea breeze and occasional tropical cyclones, were less adequately resolved. Winds during the major spawning period of mid October to December were predominantly from the west.

Temperature and salinity fields around the lateral boundaries of the model were interpolated from a global circulation model known as the Australian Community Ocean Model or ACOM (Schiller et al. 2000). In the absence of reliable surface fluxes, interior temperatures and salinities were modified by relaxing them towards ACOM values with a 10-day relaxation timescale. Sea levels on the boundaries were also taken from ACOM output, with the addition of tidal constituents derived from tide gauge observations around the Cape Cuvier and Bonaparte Archipelago areas in combination with global tidal model estimates along the offshore boundaries (Eanes & Bettadpur 1995).

### Particle Dispersion and Tracking

The modeled currents provided an indication of the instantaneous movements of larvae in the water column. However, additional information was required to estimate advection and dispersion patterns. Individual-based particle-tracking techniques were adopted for this purpose. In the absence of any detailed information on larval swimming behavior, all particles were assumed to be nonmotile and neutrally buoyant. These assumptions are relatively easy to justify in the extremely energetic environment of Eighty Mile Beach, where tidal currents and turbulent mixing velocities would normally be expected to far exceed realistic larval swimming speeds (e.g., Condie 1999).

A large number ( $\sim 10^5$ ) of neutrally buoyant particles were initially seeded randomly through the water column across the model domain, with highest concentrations on the inner shelf. The circulation and particle movement calculations were then conducted simultaneously, with particle positions being updated every 10 min by the interpolated model current velocities. A random walk component was also added to the trajectory to represent the dispersive influence of turbulent motions not resolved by the circulation model. Each particle was individually tracked and its location recorded every 3 hours.

Particles followed complex paths that were sensitive to their initial location. Individual trajectories therefore provided a limited view of likely dispersion patterns associated with large numbers of larvae. A statistical description of the dispersion results was therefore developed from large numbers of trajectories (Condie et al. 2005b). From the spawning areas defined by the MOP data, larvae were tracked over their estimated pelagic phase of 24–31 days within the main spawning period of mid October to late December (Rose et al. 1990). More specifically, particles within the spawning areas (defined later) at midnight on October 15 were tracked for 24 days, after which their locations were recorded every 3 hours over

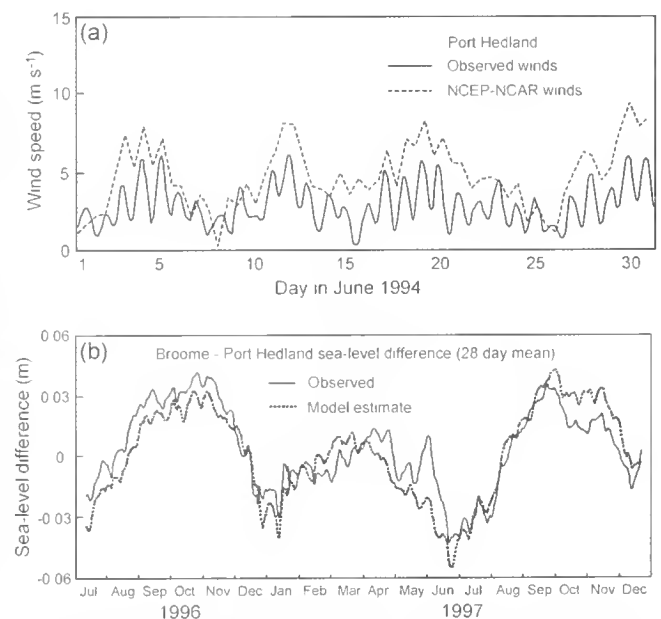


Figure 2. (a) Comparison between local observed winds at Port Hedland and the NCEP-NCAR winds used to force the model for June 1994. (b) Comparisons of observed and modeled low-frequency sea-level difference between Broome and Port Hedland, based on a 28 day running mean.

the following 7 days. The process was then repeated starting 3 h later (i.e., 3:00 AM on October 15) and then at all subsequent 3 hourly steps until the end of the 31-day pelagic phase corresponded to December 31. To ensure that the results were not unduly dominated by those particles remaining within spawning areas for extended periods, each particle was only permitted to initiate a new trajectory 7 days after it had initiated the last one.

The combination of all recorded locations provided the final estimate of potential settlement distribution. This differs from an estimate of actual settlement, which would require additional factors such as habitat distribution, habitat preference, and larval mortality to be taken into account. The approach taken to estimate spawning areas defined by the spat data was the same as that described above, except that trajectories were tracked back in time. Results were obtained for all modeled years (1994 to 1999), which were further amalgamated into an average distribution over the entire 5-year modeling period.

#### Biological Surveys

A pearl oyster stock survey conducted in September 2001 revealed that distributions are largely limited by habitat availability (Hart & Friedman 2004). This finding, combined with the longevity of the large broodstock (or MOP) (Rose et al. 1990), suggests that distributions are likely to be relatively stable and hence that the 2001 MOP distributions represented an acceptable proxy for the modeling period (1994–1999). A measure of the relative abundance of MOP was based on the average number of shell collected for each hour the vessel drifted within the target populations (Hart & Friedman 2004).

Spat surveys (measuring 0+ and 1+ age classes) were undertaken over the 3-year period from 2001 to 2003 using the method described in (Hart & Joll 2005). Because only partial coverage was provided by each survey, the three were combined here into a single estimate of settlement distribution. In brief, the spat methodology involved examining adult shell collected by commercial divers, and counting any attached spat (Hart & Joll 2005). These counts were assumed to be representative of relative spat densities across all suitable habitats available in the area. The average number of spat per shell within a 10-m grid cell therefore provided a measure of spat relative abundance. Information on the spat year classes were not required for the current analysis, however were useful for evaluating year-to-year trends in settlement.

## RESULTS

#### Modeled Currents and Sea Levels

Instantaneous current patterns on the NWS were dominated by strong tides, with speeds approaching  $2 \text{ m s}^{-1}$  during the spring tide (Fig. 3a). In the main fishing areas around Eighty Mile Beach, the tidal movements were predominantly in the cross-shore direction and diminished with distance offshore. The model currents have been shown to compare favorably with observations around the Dampier region to the west (Condie et al. 2005a). However, no direct current observations have been taken in the Eighty Mile Beach region (Godfrey & Mansbridge 2000). The best indication of model performance in the local region was therefore provided through comparisons of sea level variability.

Sea level was strongly dominated by the very large tidal signal, with ranges up to 10 m in King Sound. The model generally

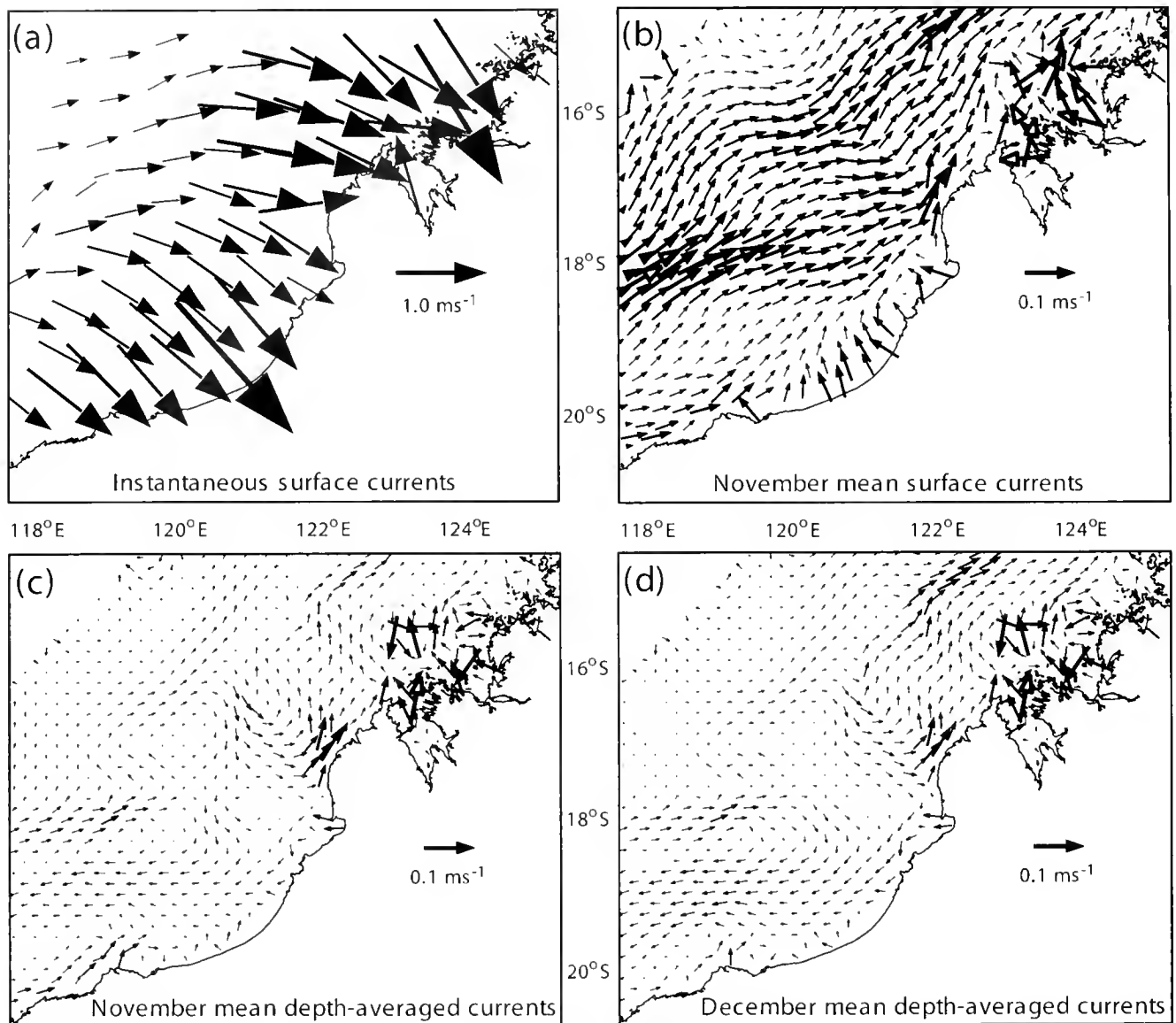
reproduced the tidal sea-level signal quite accurately, with correlation coefficients increasing from  $r = 0.87$  at Broome to  $r = 0.96$  at Port Hedland (Condie et al. 2005a). However, it is the lower frequency component that largely controls net transport over the pelagic larval phase (24–31 days). Because subtidal currents are generally highly correlated with subtidal sea-level differences, model performance in the Eighty Mile Beach region was examined in terms of sea-level differences between Broome and Port Hedland. This analysis revealed a strong coastally trapped wave signal in the model and observations, with a period of approximately 14 days in both instances. However, it is the variability over the typical larval phase that is of most immediate interest. For example, comparisons of the running mean of the sea-level difference over 28 days produced a correlation of  $r = 0.89$  (Fig. 2b).

Monthly averaged surface currents during the period October to December (main spawning period) were typically northeastward at a few centimeters per second (Fig. 3b). However, close to Eighty Mile Beach the surface flow was directed offshore, suggesting a net upwelling over the monthly timescale. These currents patterns were a response to the prevailing westerly winds and are consistent with the few satellite tracked ocean drifters observed in the area at this time of the year (Cresswell et al. 1993). The depth-averaged currents were weaker and tended to form a clockwise gyre off Eighty Mile Beach (Fig. 3c). The resulting flow close to the coast was predominantly westward, counter to the prevailing winds. This monthly averaged pattern persisted throughout the spawning period (Fig. 3d).

#### Estimates of Settlement Distribution

The surveyed MOP distribution (Fig. 4a) showed low to moderate relative abundances ( $0\text{--}60 \text{ h}^{-1}$ ) around the 10 m depth contour, with a tendency towards higher values at the northern extremity of the sampling area ( $60\text{--}120 \text{ h}^{-1}$ ). However, the largest abundances ( $110\text{--}160 \text{ h}^{-1}$ ) were found in a patch further offshore around the 30-m depth contour. Potential larval settlement distributions were estimated by assuming that MOP survey abundances were proportional to the spawning population (although results were qualitatively similar when all cells with surveyed MOP were given equal weighting).

Results averaged across the model runs (1994 to 1999) indicated two main potential settlement areas (Fig. 4b). The larger was centered just inshore of the 10 m depth contour ( $121^{\circ}24'E$ ,  $19^{\circ}6'S$ ), with the smaller centered just inshore of the 20 m depth contour ( $121^{\circ}7'E$ ,  $19^{\circ}11'S$ ). Because larvae could potentially settle at any time during the tidal cycle, the size of these areas was largely determined by the dimensions of the tidal ellipse. The relative settlement rate in the two areas varied from year to year and in 1994 the model results showed more settlement offshore than onshore (not shown). However, the predicted settlement areas were not associated with the two distinct spawning areas. Rather, most MOP regions contributed to both settlement areas (C to F in Fig. 4a and Fig. 5a) reflecting temporal and spatial variability in flow patterns within the 2.5-month spawning period. The main exception to these trends was the surveyed area near the 30 m depth contour, whose modeled larvae tended to move into deeper water ( $\geq 40 \text{ m}$ ) to the southwest without contributing to either of the main settlement areas (A in Fig. 4a and Fig. 5a).



**Figure 3.** (a) Example of flood-tide currents under spring-tide conditions. (b) Monthly mean surface currents (depth = 1.5 m) for November based on 6 years of model runs (1994–1999). (c) Monthly mean depth-averaged currents for November based on 6 years of model runs (1994–1999). (d) As in (c) for December.

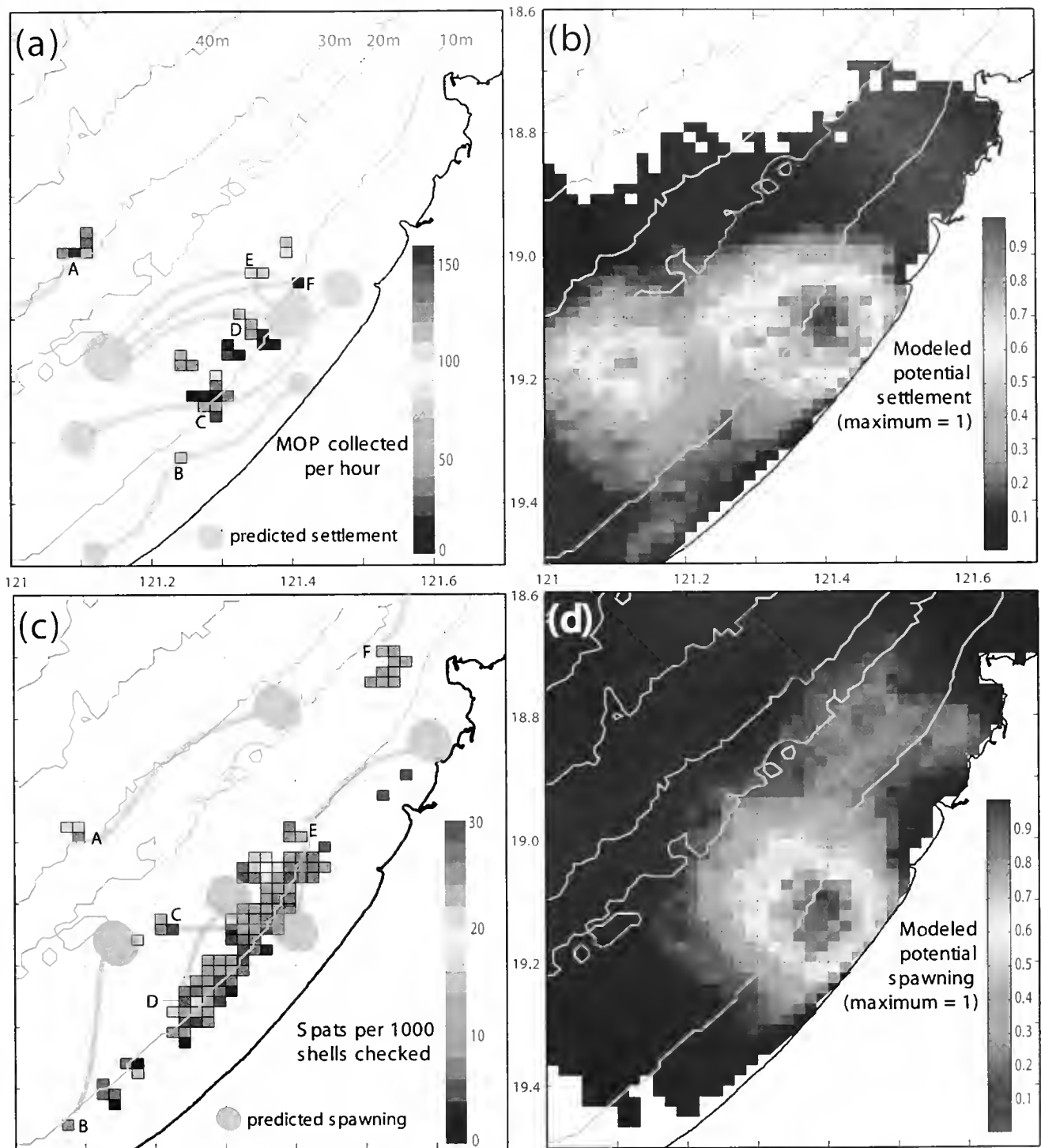
The model predicted settlement region covers nearly all the areas surveyed for spat, with predicted high settling rates corresponding with the main survey area (cf. Fig. 4b and 4c). However, spat surveyed further offshore and to the north (Fig. 4c) corresponded to low settlement rates and may have originated from MOP outside the MOP survey area. There were also large areas of predicted settlement, both near-shore and offshore, that were not in the area of the spat surveys. Whereas spat may have existed in some of these areas, the limited availability of suitable habitat would likely have resulted in low abundances.

#### *Estimates of Spawning Distribution*

The surveyed spat distribution, as described by Hart and Joll (2005), tended to follow the 10-m depth contour along Eighty Mile Beach at low to medium relative abundances (0–20 per thousand shells) with a bias towards deeper water to the north (Fig. 4c). There were a few high abundance of cells (20–30 per thousand

shells) in this zone, with more in habitats further offshore around the 15-m and 30-m depth contours (Fig. 4c). Potential spawning distributions were estimated by assuming that the spat survey abundances were proportional to the settling population (although results were qualitatively very similar when all cells with surveyed spat were given equal weighting).

Estimated spawning areas associated with the surveyed spat distributions were centered just inshore of the 10-m depth contour ( $121^{\circ}24'E$ ,  $19^{\circ}6'S$ ), but relatively high values extended offshore almost to the 20-m depth contour and onshore almost to the coast (Fig. 4d). The size of these areas was again a function of the tidal ellipse and there was very limited interannual variability (not shown). Spat counted in the main survey area along the 10-m depth contour were mainly spawned in this area (C, D and E in Fig. 4c and 5b), although cell E also received model larvae from further to the northeast. The model indicated that spat surveyed further south (B) and north (F) would have come from spawning in deeper water, whereas those offshore around the 30-m depth contour (A)



**Figure 4.** (a) Relative abundance of MOP off Eighty Mile Beach expressed in terms of the number collected per hour of vessel drift during the survey (Hart & Friedman 2004) overlain by a schematic summary of modeled downstream settlement areas from selected cells A to F (corresponding quantitative predictions for A to F are shown in Fig. 5a). (b) Estimated settlement distribution (ignoring habitat availability) averaged across all model years (arbitrary units with maximum = 1). (c) Relative abundance of spat off Eighty Mile Beach expressed in terms of average number of spats found per 1,000 shells checked (Hart & Joll 2005) overlain by a schematic summary of modeled upstream spawning areas from selected cells A to F (corresponding quantitative predictions for A to F are shown in Fig. 5b). (d) Estimated spawning distribution averaged across all model years (arbitrary units with maximum = 1).

would have come from marginally shallower depths to the north-east (Fig. 4c and 5b).

Apart from extending into very shallow water, the spawning area estimated from the surveyed spat (Fig. 4d) was consis-

tent with the surveyed MOP distribution (Fig. 4a). The main exception was the offshore MOP (A in Fig. 4a), which the model suggests spawn into deeper water outside of the spat survey region (Fig. 5a).



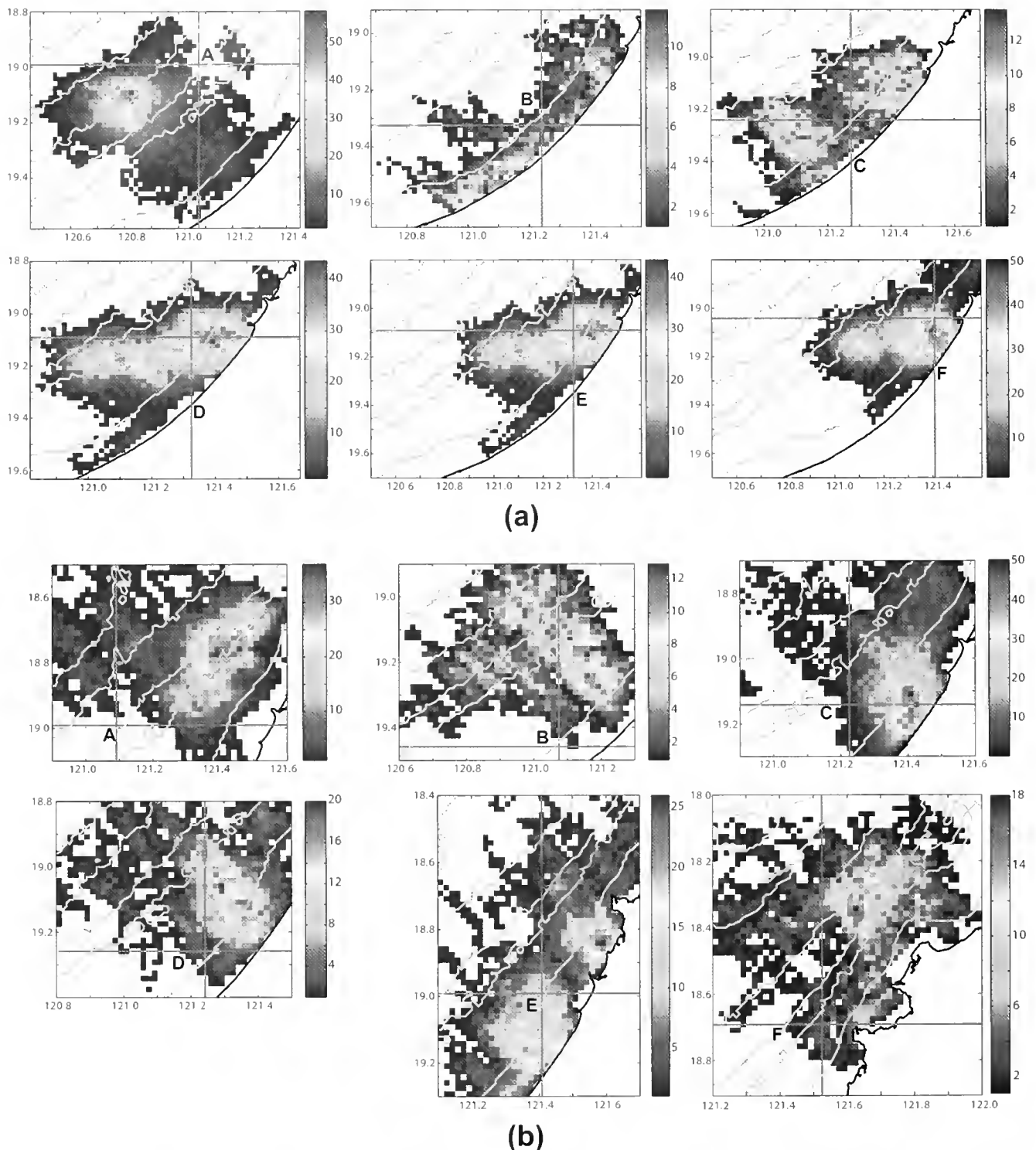


Figure 5. (a) Relative likelihood of settlement after spawning at the cell marked by the cross-hairs. (b) Relative likelihood of spawning contributing to settlement at the cell marked by the cross-hairs. In both (a) and (b) results are averaged across all model years (1994–1999), the color-bars indicate the number of trajectories connecting a given cell with the cell marked by the cross-hairs, and depth contours are at 10-m intervals.

## DISCUSSION

The overall picture emerging from the model results is that spawning in the Eighty Mile Beach region is concentrated around the surveyed MOP distribution between 8- and 15-m depth, with

potential smaller contributions from further northeast. These spawning events are likely to lead to successful recruitment locally and alongshore to the southwest so that the main pearl oyster producing populations are likely to be self seeding. These spawning events also feed larvae into neighboring shallow coastal envi-



ronments (through tidal oscillations) and deeper waters to the west (~20 m). However, spat abundances appear to be lower in these areas, suggesting that recruitment is either limited by habitat availability and possibly increased mortality in shallow water, or else spat larvae preferentially select other settlement habitats (as opposed to live adult shell). High local abundances of MOP and spat observed in deeper water (~30 m) appear to be supported by intermittent larval transport from inshore populations and may be further assisted by high quality habitat and low mortality rates. However, spawning in this area seems to contribute little to recruitment in the inshore populations.

The validity of the settlement and spawning estimates is dependent on a number of factors. The first is the accuracy of the predicted circulation. Whereas the model has been shown to perform relatively well in the Dampier region (Condie et al. 2005a), the data available for validation near the fishing grounds was limited to sea-level information. The second factor is the comprehensiveness of the MOP and spat surveys. For example, some habitats may have been under sampled and others may still be undiscovered.

A third source of model uncertainty is associated with larval behavior. For example, larvae remaining close to the surface would tend to follow the wind dominated circulation to the north-east (Fig. 3b), rather than the depth averaged circulation (Fig. 3c). This seems unlikely in this region given that tidal motions drive energetic vertical mixing out to at least the 30-m depth contour and that the MOP and spat surveys suggest transport to the southwest. A potentially more significant behavioral characteristic would be

any ability of larvae to select settlement sites. During the week long period in which settlement can occur (Rose et al. 1990), tidal motions carry individual larvae back and forward up to 20 km in the cross-shore direction. If larvae encountered preferred habitats over depths from around 8–15 m deep during this period, then the estimated settlement distribution based on the observed MOP (Fig. 4b) would be very similar to the observed spat distribution (Fig. 4c).

The results of the model suggest that the long-standing hypothesis within the pearling industry, namely that deeper “unfished” stocks are a broodstock source for the commercially fished inshore stock, is not likely to be true. The inshore stocks appear to be self-sustaining, and may even be providing larvae to deeper stocks in irregular recruitment events. These findings have important management implications, particularly if fishing for MOP were to recommence at some time in the future. However, current management and fishing strategies seem to protect the critical broodstock that support the long-term sustainability of the existing “gauntlet” fishery.

#### ACKNOWLEDGMENTS

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## EFFECTS OF SEASON, TEMPERATURE CONTROL, BROODSTOCK CONDITIONING PERIOD AND HANDLING ON INCIDENCE OF CONTROLLED AND UNCONTROLLED SPAWNING OF GREENLIP ABALONE (*HALIOTIS LAEVIGATA* DONOVAN) IN WESTERN AUSTRALIA

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**ABSTRACT** Managing the broodstock conditioning process is important to the successful establishment of an abalone industry that can reliably produce juveniles from captive stock and pursue selective breeding. Four conditioning periods of 6, 8, 10 and 12 wk intervals were tested with captive greenlip abalone (*Haliotis laevigata*), using a constant conditioning temperature and ambient temperature as a control. The conditioning periods of 6, 8, 10 and 12 wks are equivalent to ~420, 560, 700 and 840 EAT (effective accumulative temperature), based on a biological zero point (BZP) of 6.9 °C for gonad development. Greenlip abalone broodstock collected from the wild (87.5–142.1 mm in shell length and 108.2–482.8 g in whole weight) were spawned in all seasons including numerous induced spawnings outside of the normal breeding period with conditioned stock. Over the whole trial period, greater egg production from prescheduled spawnings occurred in the conditioned group with an average of  $1.70 \times 10^6$  eggs per tub of 5 female abalone per planned spawning, compared with abalone held in ambient control tubs that only produced an average of  $0.40 \times 10^6$  eggs per tub. Animals that were spawned every 8 wks produced the largest average number of eggs per holding tub, however, there was very little difference between conditioning periods in terms of number of successful spawnings per spawning round. The egg production for all planned spawnings was highest before the “natural spawning season” for greenlip abalone. In comparison, the highest numbers of unplanned spawnings occurred around the natural spawning period for the conditioned and control groups. Handling abalone did reduce egg production within the trial, but this was not statistically significant. Histological examination showed that using the visual gonad index is not a good indication of maturation of the abalone and confirmed that abalone can be conditioned out of season.

**KEY WORDS:** *Haliotis laevigata*, broodstock conditioning, abalone, handling, conditioning periods, gonad histology.

### INTRODUCTION

Aquaculture of abalone is practiced in many countries worldwide and in Australia is an emerging industry across the southern mainland states and Tasmania (Fleming 2000). However, little research has been carried out in Australia on conditioning of mature abalone for use as broodstock. Understanding the broodstock conditioning process is important to the successful establishment of this industry by ensuring reliable and cost effective production of juveniles and for selective breeding (Li 2001). The ability to have ripe individuals year-around or at predictable times in the year allows the culturist to schedule the hatchery process to occur at the most favorable time of the year for growth and survival (Hahn 1989). The use of conditioned abalone overcomes problems relating to the use of wild-caught adult abalone for broodstock including access to stock (through state regulations), variability in the condition of stock and the reliability of successful spawnings (Fleming 2001).

The major species farmed in Western Australia is the greenlip abalone *Haliotis laevigata* Donovan. This species is distributed along the southern coastline from western Victoria to Cape Naturaliste in Western Australia and around islands near Tasmania (Freeman 2001). Broodstock conditioning research has been conducted on this species in that state (Daume & Ryan 2004) and on the genetically distinct population (Elliot et al. 2001) in Tasmania, where ambient temperatures are much lower than in Western Australia and exhibit greater seasonal variation (Leonart 1992, Grubert & Ritar 2002, 2003, Freeman 2001). One of the major problems in investigating abalone reproduction is the short period of natural spawning (Hahn 1989). The natural spawning

season for greenlip abalone on the south coast of Western Australia is from late September/early October through to December (Wells & Mulvey 1992).

Several biological processes are involved when conditioning abalone, for example, gametogenesis for females and males, synthesis of hormones controlling gametogenesis and spawning, and for the female only, vitellogenesis and synthesis of hormones controlling vitellogenesis. In addition to the gonad being ripe, the animal must be in the proper physiological state and in appropriate environmental conditions to promote spawning (Hahn 1989). Many factors have been found to influence conditioning in different species of abalone including water temperature, food, photoperiod or a combination of these variables (Hahn 1989). Uki and Kikuchi (1982) considered nutrition as an important factor for gonad development in abalone and Webber (1970) found that water temperature controls the reproductive cycle for many marine gastropods. In most species of abalone, temperature strongly influences rates of gonad development; its effect is cumulative above a certain threshold temperature and is known to vary between species (Grubert & Ritar 2003). The threshold temperature, better referred to as the “biological zero point” (BZP) was first identified by Kikuchi and Uki (1974). They determined the effective accumulative temperature in degree-days (EAT° C-days) for gonad conditioning of *Haliotis discus hamai* Ito and *Haliotis discus* Reeve by subtracting the BZP from the daily water temperature and adding this figure over the conditioning period (in days). *H. discus hamai* was found to have a BZP of 7.6°C (Uki & Kikuchi 1984). Grubert and Ritar (2003) studied the effect of temperature and conditioning interval on the spawning success of wild-caught blacklip (*Haliotis rubra* Leach) and greenlip abalone fed an artificial diet. They found that unlike *H. rubra*, male and female *H. laevigata* have a higher spawning rate and gamete production when conditioned at 16°C compared with 18°C. In a preceding

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experiment, Grubert and Ritar (2002) estimated BZP values for gonadal and larval development for *H. rubra* as 7.8°C and 7.9°C respectively and for *H. laevigata* 6.9°C and 7.2°C respectively. In Leonart's (1992) research into broodstock conditioning of green-lip abalone, the mean temperature during conditioning was 16°C and the number of elapsed degree-days was recorded as 1,750, which when corrected using a BZP of 6.9°C gives an EAT of 995 degree days. After this time, the abalone were only just coming into condition. For this study on broodstock conditioning in green-lip abalone, a BZP of 6.9°C was assumed.

The aim of this study is to develop a sound protocol for conditioning broodstock abalone for commercial purposes by determining the most favorable conditioning period (e.g., 6, 8, 10 or 12 wks) as indicated by a visual condition index, histological assessment, spawning success rate (as a % of the group subjected to spawning induction), fertilization rate and hatch rate. Seasonal patterns for controlled and uncontrolled ("wildcat") spawnings and whether handling stress influences the success of broodstock conditioning and initial spawning success were also investigated. Gonad histology and macroscopic observation of the gonad were assessed as relative guides to the likelihood of spawning success.

## METHODS

The term "conditioning" is used here for the process adopted to induce gonad ripeness and ability to spawn in captive adult abalone, usually outside of the normal spawning season but also to allow multiple spawnings within that season.

### Experimental Set-up

The research was conducted at a commercial hatchery, Great Southern Marine Hatcheries (GSMH), Albany (117°57'38"E, 35°5'52"S), Western Australia. Wild abalone were collected from Bremer Bay, approximately 200 km east of Albany, and held in the conditioning system for up to 7 mo before the trial commenced, where broodstock were acclimatized and temperature control was refined. The trial ran for 36 wks from April 2001 to December 2001. The culture system consisted of 60, 30-L round plastic tubs, with 8 male tubs and 24 female tubs at 17.0°C  $\pm$  0.4°C in the conditioning room, and 8 male and 8 female control tubs in an outdoor grow-out area to provide ambient conditions (e.g., natural photoperiod and temperature with unfiltered sea-water). The remaining 14 tubs of females were used for histology and were also held in the conditioning room at 17.0°C  $\pm$  0.4°C. Each tub contained 5 abalone and males and females were held in separate tubs. The abalone used in this experiment were of mean shell length 118.1 mm mean size (range 87.5–142.1 mm total shell length) and 250.4 g whole weight (108.2–482.8 g).

The continuously aerated tubs had a flow rate of 1–1.5 L/min ( $\approx$  2–3 water exchanges per hour) and a central standpipe. Water supplied to the reservoir (5,000 L) and heater/chiller unit was filtered down to 10  $\mu$ m using a bag filter. A photoperiod of 12 h low light and 12 h darkness was used for animals that were kept indoors.

### Spawning Groups

All of the tubs except those set aside for histological examination were randomly assigned into 4 groups (6, 8, 10 and 12 wk spawning intervals). This is equivalent to  $\approx$ 420, 560, 700 and 840 EAT based on a BZP of 6.9°C. Over a 36-wk period, groups were spawned at 6, 8, 10 and 12 wk intervals respectively (Table 1).

TABLE 1.  
Spawning schedule for each group of abalone.

Group	Spawning weeks (from day 0*)					
6 Week	6 (May)	12 (July)	18 (Aug)	24 (Sept)	30 (Nov)	
8 Week	8 (June)	16 (July)	24 (Sept)	32 (Nov)	—	
10 Week	10 (June)	20 (Aug)	30 (Nov)	—	—	
12 Week	12 (July)	24 (Sept)	36 (Dec)	—	—	

\* Day 0 was the day when an attempt was made to spawn out all abalone after collection.

Each spawning group consisted of 6 female tubs and 2 male tubs from the conditioning room and 2 female tubs and 2 male tubs under ambient conditions (controls).

All animals were induced to spawn following commercial spawning protocols without being removed from their holding tubs. This strategy was aimed at minimizing handling of the abalone. Therefore for all spawnings, egg production was recorded per tub of 5 female abalone not per individual female. Animals from the histological examination group were not deliberately induced to spawn during the trial. Two tubs of females from this group were sacrificed every 4 wk for analysis by histology (see below).

Spawning success (%) was calculated for each planned spawning by:

Number of tubs of abalone that spawned  $\times$  100  $\div$  Total number of tubs of abalone that were subjected to spawning induction stimuli

Unplanned ("wildcat") spawnings also took place. As with data for planned spawnings in which the number of spawning inductions depended on the conditioning interval (6–12 wk), estimates of incidences of spawning and egg production had to take into account the number of tubs for each treatment (conditioned or control). Similarly data analysis by season also was corrected for the number of days of each season (Spring, Summer, Autumn, Winter) encompassed within the 36-wk trial.

Wildcat spawnings (%) for each month was calculated by:

Number of tub  $\times$  day combinations per month with evidence of wildcat spawnings  $\times$  100  $\div$  Number of days in month  $\times$  Number of tubs for control (=16) or conditioned (=32)

Similarly, wildcat spawnings (%) per season for each conditioning period and controls was calculated by:

Number of tub  $\times$  day combinations per month with evidence of wildcat spawnings  $\times$  100  $\div$  No of days in season  $\times$  Number of tubs for conditioning group (=8) or controls (=4)

### Feeding and Cleaning

All individuals were fed daily *ad libitum* with a commercial formulated, extruded conditioning diet (Adam and Amos Feeds, South Australia). Feed was given as a rate of 1% to 2% body weight per day.

Each tub of abalone were cleaned daily (morning) with as little disturbance as possible. Complete drainage of each tub occurred three times a week. Every other day, tubs were just siphoned clean to minimize stress related to cleaning procedures. Rinsing water for the tubs was maintained at the same temperature as the culture vessels to eliminate possible spawnings from temperature shock.

### Histology

Histological assessment was carried out on female greenlip abalone to determine progress with conditioning. Egg development was assessed every 4 wks, over a period of 24 wks, commencing April 11, 2001 by sacrificing two tubs of female abalone from the histological examination group and fixing them whole in 10% neutral buffered formalin. Gonad samples from the fixed samples for histology were cut from the abalone, dehydrated, embedded in paraffin wax, sectioned at 5  $\mu\text{m}$  and stained with hematoxylin and eosin using standard techniques. The gonads were examined and classified using the stages described in the abalone atlas produced by Handlinger et al. (2001). The stages they describe are 1 = Recovery phase gonads; 2 = Growing phase gonads; 3 = Mature ovaries; 4 = Atretic ovaries and 5 = Resting phase ovaries.

### Handling to Assess Gonad Index

Throughout this experiment, abalone in the conditioning room, except those used for visual gonad index (below) and histology assessment, were never removed from their tubs. These tubs were used as the nonhandled control group for the assessment on handling versus nonhandling.

Every 14 days after the start of the experiment, two female tubs from each spawning group (6, 8, 10, 12 wk) were weighed, measured and visually assessed to determine the level of gonad ripeness. The same two tubs of animals were chosen each time to assess gonad ripeness, growth data over time and for the effects of handling. Abalone were removed by lifting them off the bottom with a spatula-like tool. The progression of gonad development was determined for each individual using a visual scoring system of 0–3 and was referred to as the visual Gonad Index (GI). A score of 0 = cannot determine the sex, 1 = the upper edge of the gonad was below the rim of the shell, 2 = the upper edge of the gonad was even with the rim of the shell, and 3 = the upper edge of the gonad was above the rim of the shell. This means that at a score of 1 the sex of the abalone can be determined, at a score of 2 there is a possibility the abalone may spawn and at a score of 3 it is assumed the abalone is highly likely to spawn. The egg production results were used to assess the influence of handling on spawning success, in comparison with the remaining 4 tubs of females from the conditioning room for each spawning group.

### Experiment Initiation

All broodstock abalone used in the experiment were tagged and initial weight and shell length data were recorded. To initiate the experiment commercial spawning methods were used to induce spawning. All abalone were exposed to 18.5°C water temperature for 12 h, then desiccated for 45 min followed by an ultra violet (UV) treated water temperature shock of 21°C, which was slowly decreased back to 17°C over a period of 8 h. Animals were held in the UV treated water for a maximum of 24 h to induce spawning. After this time they were returned to their normal experimental conditions in the conditioning room or control area. The GI for each abalone were assessed and recorded during the desiccation time.

### Data Recording

A temperature data logger was placed into the reservoir. Daily temperatures were also recorded manually. Water quality was checked weekly (DO, pH, ammonia and salinity). Atmospheric pressure readings were recorded on a daily basis.

Throughout the experiment any spawnings that occurred were recorded, including "wildcat" spawnings, which are spawnings that occurred unexpectedly throughout the trial. All results using data from "wildcat" spawning events excludes "wildcat" spawning data from the histology tubs. The natural spawning season for greenlip abalone was considered to be September to December. Where possible, numbers of eggs per tub and per group were recorded. Total number of eggs per batch was estimated from 3 sub-samples as:

Average number of eggs in 1-mL samples  $\times$  Total volume of the sample.

### Data Analyses

Statistical analyses were carried out using the STATISTICA 6.1 (Stat Soft, Inc. 2002) computer package. The assumptions of normality and homogeneity of variance were confirmed graphically for each data set using box plots. Egg production data between conditioned and control tubs, and handled and non-handled tubs were compared with *t*-tests. Spawning success was also compared with *t*-tests between males and females. Spawning success, egg production and histological egg stages between conditioning intervals were analyzed using 1-way ANOVA. Relationships between the wildcat spawning and daily barometric pressure were explored using simple regression analyses.

## RESULTS

### Water Temperature and Quality

Water temperatures in the conditioning room were usually constant between 16.6°C to 17.2°C, whereas temperatures in the control tubs ranged from 14.0°C to 20.2°C (Fig. 1). Dissolved Oxygen ranged from 76.9% to 105.0% (mean 90.0%); pH was within the range of 7.78–8.29 (mean 8.17), whereas salinity was recorded between 35.2 ppt and 36.0 ppt with a mean of 35.5 ppt. Free ammonia concentrations were less than 0.1 mg  $\text{NH}_3\text{-N/L}$ .

### Survival and Growth

Excluding abalone for histological assessment, high survival (>97%) was observed throughout the trial for all greenlip abalone held in the conditioning room and control area.

Mean average shell length for the conditioned and control tubs of abalone over the whole trial period of 36 wks was calculated at 6.03  $\mu\text{m/day}$  and 5.07  $\mu\text{m/day}$  respectively, whereas the mean

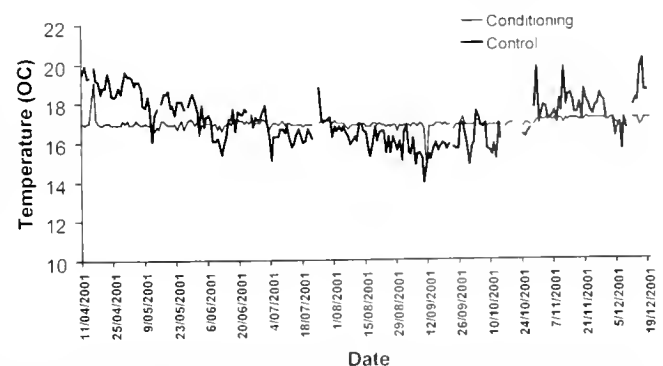


Figure 1. Average water temperatures in broodstock tanks in the conditioning room and in the ambient control area throughout the trial.

average weight gain per abalone for the conditioned and control tubs of abalone, were 30.80  $\mu\text{g/day}$  and 31.82  $\mu\text{g/day}$  respectively.

### Spawning

In initiating the experiment no abalone actually spawned. However, wildcat spawnings (unplanned spawnings) had occurred in the majority of tubs within the 2 wks before initiating the experiment.

Wildcat spawning data for abalone held in the control groups showed that in April, outside of the natural spawning season, there were some wildcat spawning events occurring, whereas in May through to August, again outside the natural spawning season, there were very few wildcat spawning events (Fig. 2). However, in September through to December, during the natural spawning season, many more wildcat spawnings were recorded in both conditioned and control animals. For the planned spawning groups (every 6, 8, 10 and 12 wks), all wildcat spawning data showed similar patterns (Fig. 3). However, it was evident that more wildcat spawning events occurred in summer, within the natural spawning season in the controls (Fig. 3) despite the trial covering only 1 month in summer.

Planned spawning results showed that, over the whole trial period, egg production in the conditioned abalone was significantly greater than egg production in the controls ( $df = 28$ ,  $t = 2.299$ ,  $P = 0.029$ ). Conditioned abalone produced 1.70 million eggs per female tub per planned spawning compared with abalone held in the control tubs that only produced an average of 0.40 million eggs per female tub (Fig. 4). Highest egg production was recorded within the winter months, outside the natural spawning season for abalone held in the conditioning room with an average of 3.16 million eggs per tub of 5 female abalone per planned spawning (Fig. 5). In comparison, for abalone held in the control tubs, the highest average egg production of 0.87 million eggs per tub of female abalone was recorded during spring (Fig. 5). Egg production from planned spawnings was lowest from abalone held in the conditioning room during the summer months (Fig. 5).

Results for the spawning intervals of 6-, 8-, 10- and 12-wk periods showed that abalone from the week 8 spawning interval had the highest egg production during the second spawning induction (week 16) of 44.23 million per tub of female abalone (Fig. 6). Egg production was very low in the first planned spawning attempt for spawning intervals of 6 and 8 wk of 2.5 million and 1 million respectively. However, spawning occurred in only about 13% of female tubs of abalone spawned during both inductions. For

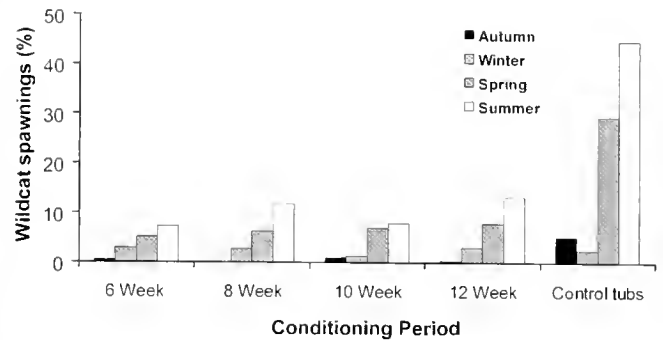


Figure 3. Percentage of unplanned ("wildcat") spawning events that occurred in each spawning interval (6, 8, 10 or 12 wk) and control tubs of greenlip abalone for each season throughout the trial

spawning intervals of 10 and 12 wks the opposite was observed, with highest egg production of 23 million and 19 million occurring within their first planned spawning attempt and a decline in egg production for the following planned spawning attempts (Fig. 6). During the first planned spawnings, the percentage of female tubs in which abalone spawned were 75% and 38% for spawning intervals 10 and 12 wks respectively. For the latter inductions for these spawning intervals, where egg production declined, the percentage of actual spawnings also declined to 38% and 25% in both spawning intervals for the second and third spawning attempts.

Overall, the 6 wk spawning interval had the highest average percentage spawning success of 60%, whereas the 12-wk spawning interval had the lowest of 33% (Fig. 7). However, there was no significant difference between spawning intervals for spawning success ( $df = 3$ ,  $F = 0.164$ ,  $P = 0.917$ ).

Average numbers of eggs produced for each interval showed week 8 to be slightly higher than intervals of 6, 10 and 12 wks with an average egg production of 12 million eggs produced per tub of 5 female abalone (Fig. 8). However, overall there was no significant difference between spawning intervals in terms of eggs produced per tub of female abalone ( $df = 3$ ,  $F = 0.150$ ,  $P = 0.928$ ).

There was no significant difference ( $t = 1.18$ ,  $df = 38$ ,  $P = 0.25$ ) between the percentages of males and females that spawned in each planned spawning induction.

No relationship was observed between wildcat spawning events and average daily barometric pressure ( $r = 0.06$ ) or between wildcat spawning events and change in daily pressure ( $r = 0.01$ ).

Fertilization and hatch rates were consistent between each spawning group throughout the trial. In general, all lines produced

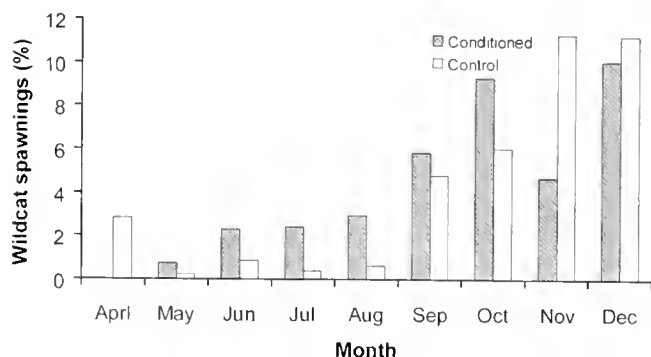


Figure 2. Incidence of unplanned ("wildcat") spawning events that occurred in the conditioning and control tubs of greenlip abalone for each month of the trial.

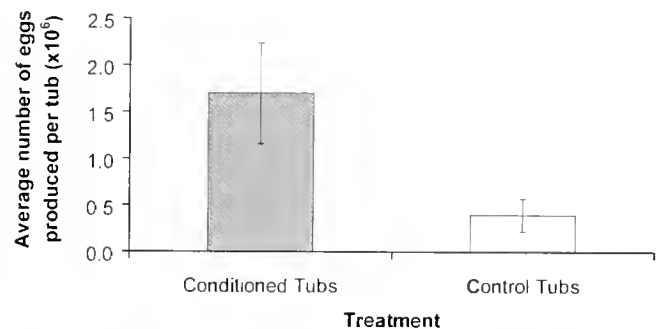


Figure 4. Average number of eggs produced ( $\times 10^6$ ) per tub of female greenlip abalone per planned spawning for the conditioned and control tubs of abalone over the trial period.

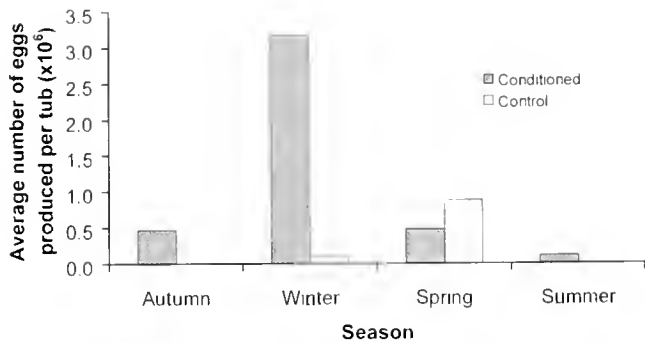


Figure 5. Average number of eggs produced ( $\times 10^6$ ) per tub of female abalone per planned spawning for conditioned and control greenlip abalone for each season over the trial period.

from conditioned and control stock displayed very high fertilization (90% to 100%) and hatch rates (80% to 100%).

#### Histology

Egg stages expressed as percentage of mature (stage 3) gonads from the histological examination results were not comparable to the results obtained for GI (Table 2). However, egg stages over time showed a significant difference ( $df = 6$ ,  $f = 4.654$ ,  $P < 0.001$ ). A significant drop was observed for the average egg stage between 0 and 4 wks ( $P = 0.005$ ), whereas a significant increase for the average egg stage was evident between 4 and 16 wks ( $P = 0.019$ ) as well as between 4 and 20 wks ( $P < 0.001$ ).

#### Handling

Overall averages for egg production for the whole trial showed that nonhandled abalone produced more eggs ( $2.06 \pm 0.68$  million per tub of female abalone per planned spawning) than abalone from the handled group ( $0.98 \pm 0.29$  million) (Fig. 9). However, this difference was not significant ( $df = 28$ ,  $t = 1.459$ ,  $P = 0.156$ ). Percentage spawning success rates for nonhandled and handled abalone were very similar and not significantly different, with values of 55% and 57% ( $df = 28$ ,  $t = 0.14$ ,  $P = 0.889$ ).

### DISCUSSION

#### Water Temperature and Quality

The broodstock conditioning system used provided stable water temperature and high water quality. In Albany, ambient water

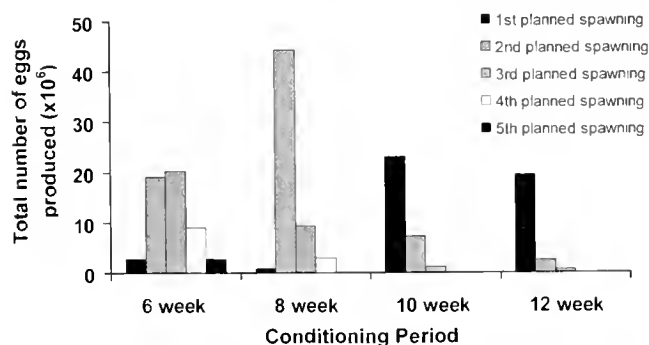


Figure 6. Total number of eggs produced ( $\times 10^6$ ) from planned spawnings for each conditioning period (6, 8, 10 or 12 wk) with greenlip abalone (data for handled, nonhandled and controls pooled).

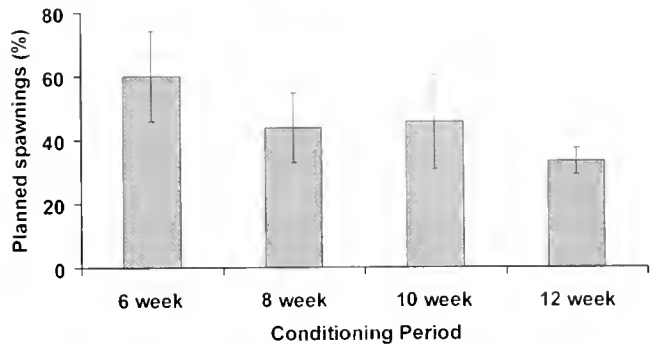


Figure 7. Average percentage of tubs of greenlip abalone, per planned spawning (mean  $\pm$  SE), in which spawning occurred for each conditioning period (6, 8, 10 or 12 wk).

temperatures exhibit a smaller range and reach much warmer temperatures than in Tasmanian waters (Hindrum et al. 1996). At warmer temperatures it is evident that abalone grow faster (Freeman et al. 2000).

In the conditioning room a water temperature increase to 19.1°C in mid April and a decrease to 14.7°C in early September can be observed in Figure 1. These fluctuations corresponded to power failures to the heater/chiller unit.

#### Survival and Growth

All broodstock abalone showed high survival (97%) but low growth rates between the start and finish of the trial. There was very little difference between mean average weight gain and mean average shell length for both the control and conditioned tubs of abalone. The overall averages of 5.77  $\mu\text{m}/\text{day}$  for average shell length and 31.54 mg/day for average weight gain for broodstock abalone are slow when compared with growth rate estimates, used for stock assessment purposes, for wild adult greenlip abalone of 25 mm/year in southern Western Australia (A. Hart pers. comm.). Moreover, they are low when compared with growth rates observed for wild juvenile greenlip abalone of 1.690  $\mu\text{m}/\text{month}$  (Shepherd 1988). Shepherd and Hearn (1983) believe that energy expenditure during gonad development can reduce growth rate. Growth rate is affected by several variables including genotype (Brown 1991), density, type and amount of feed (Day & Fleming 1992), water flow (Higham et al. 1998), water quality (Harris et al. 1998) and handling techniques such as frequency of emersion (Maguire et al. 1996).

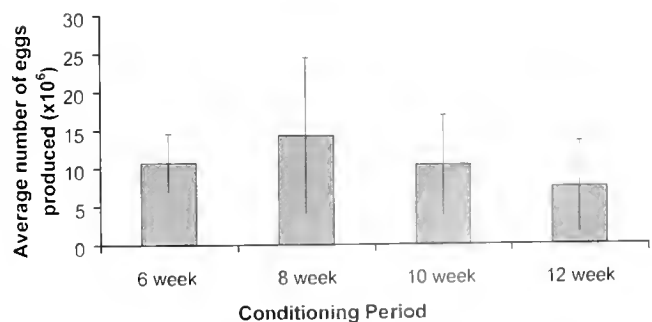


Figure 8. Average number of eggs produced ( $\times 10^6$ ) from planned spawnings (including handled, nonhandled and controls) for greenlip abalone subjected to different conditioning periods (mean  $\pm$  SE).

TABLE 2.

Gonad index (GI) and percentage of mature egg stage (stage 3) for histology tissue sections averaged for 10 abalone, which were collected every four weeks over a period of 24 weeks.

Week	Average Gonad Index*	Average % of Mature Stages (Stage 3) for Each Week Histological Samples Were Taken
0	1.15 $\pm$ 0.15	54.17 $\pm$ 0.10
4	1.40 $\pm$ 0.16	20.00 $\pm$ 0.10
8	1.10 $\pm$ 0.07	36.67 $\pm$ 0.12
12	1.10 $\pm$ 0.07	56.67 $\pm$ 0.15
16	1.20 $\pm$ 0.11	36.67 $\pm$ 0.13
20	0.89 $\pm$ 0.11	40.74 $\pm$ 0.13
24	1.00 $\pm$ 0.00	58.33 $\pm$ 0.08

\* For gonad index (GI) a score of 0 = cannot determine the sex, 1 = the sex of the abalone can be determined but spawning is unlikely, 2 = there is a possibility the abalone may spawn and 3 = it is assumed the abalone is highly likely to spawn.

### Spawning

Within the 2 wks prior to the start of this experiment, wildcat spawning occurred in the majority of tubs of abalone. This could explain why we did not get any induced spawnings when all tubs of abalone were subjected to spawning stimuli at the beginning of the experiment.

Wildcat spawning data for abalone held in the control groups followed the general pattern of a natural spawning cycle for greenlip abalone. In the warm month of April there were some wildcat spawning events occurring, but in the cooler months of May to August there were very few wildcat spawning events. However, in the warmer months of September to December in the natural spawning season for greenlip abalone a lot more wildcat spawnings were evident (Fig. 2). Shepherd and Laws (1974) found the spawning season for greenlip abalone in South Australia to be October to March, whereas Wells and Mulvey (1992) concluded that it was September to December in Western Australia.

The results presented in this study show that greenlip abalone can be spawned outside of the natural spawning season. The egg production of conditioned broodstock from planned spawnings was highest before the natural spawning season commenced (Fig. 5). Fewer successful planned spawning events and lower egg production occurred around the natural spawning season. However, a high number of wildcat spawning events occurred in all tubs dur-

ing this period. Low egg production would be expected per tub of female abalone during the planned spawnings over the natural spawning season caused by abalone already expelling their eggs in unplanned spawning events.

Spawning events before the natural spawning season can be highly beneficial to farmers because they can take advantage of the enhanced growth of juveniles during the early summer months. Animals can be weaned off the plates before the highest summer temperatures occur. High water temperature during the weaning process can cause high mortalities in some regions of Southern Australia (M. Russell pers. comm.).

The planned spawning results showed that over the whole trial period the conditioned group of abalone produced around four times as many eggs as the control groups. This could partly be explained for 2 reasons. Firstly, the control groups did not produce many eggs in winter and secondly, a lot of egg production for the control group was "wasted" on wildcat spawnings (where egg production is not recorded). However, it still strongly suggests that holding broodstock abalone in a conditioning system has a major advantage over holding broodstock at ambient conditions. In recent years, most Australian farmers have held their broodstock in ambient conditions on their farm and only increased feeding and feed type to condition their broodstock. Many studies have suggested that holding abalone at constant temperature and feeding a good quality diet will condition broodstock abalone (Kabir 2001, Grubert & Ritar 2003, Lleonart 1992, Moss 1998).

It was observed that the week 8 spawning interval produced a larger number of eggs per tub of abalone compared with all other spawning week intervals. The data for the first planned spawning for the week 8 spawning interval shows low egg production. This could suggest that most abalone in this group for the second planned spawning were actually conditioned for 16 wk and demonstrates the benefits of longer conditioning periods. This trial was only set to test a maximum interval of 12 wks. Further studies into longer conditioning times are now in progress.

### Histology

Histological assessment of egg stages in greenlip abalone is more accurate in determining the maturity of the abalone than using the visual GI scoring system.

Histology results show a significant drop between 0 and 4 wks, suggesting that spawning or reabsorption of eggs may have reduced the average egg stage for that period. The significant increase in average egg stage between 4 and 16 wks and 4 and 20 wks suggests that the abalone come into condition around this time, which equates to 12 and 16 wks for conditioning. The need for a longer conditioning period for this species was also evident in Grubert and Ritar (2003), who found for greenlip abalone in Tasmania, egg production peaked when held at 18°C for 114 days (1265 EAT°C-days). Assuming the BZP of greenlip abalone is 6.9°C (Grubert & Ritar 2002) then this equates to approximately 18 wk at our trial temperature of 17°C. In another study in Tasmania, Leonart's adjusted results of 16 wks at 17°C, also shows that a longer conditioning period is needed (Lleonart 1992).

In terms of the average number of eggs produced, weeks 6 and 8 were not very successful, which suggests that the abalone are not in condition after such short conditioning periods. Conditioning intervals of 10 and 12 wks appeared to be more successful. The first planned spawning for both the 10 and 12 wks intervals showed good egg production. The second and third planned

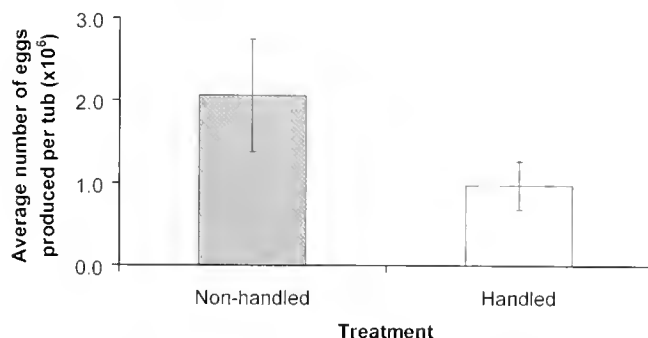


Figure 9. Average egg production ( $\times 10^6$ ) per tub of female abalone (mean  $\pm$  SE) per planned spawning for nonhandled and handled greenlip abalone (data for conditioned and control tubs pooled).



spawning events for these two groups were not as successful because of high numbers of wildcat spawning events that appear to be uncontrollable in the warmer months of the year. Clearly, greenlip abalone do need at least 10 wks for conditioning.

Generally, most farmers use the GI as an indication of the abalone's spawning ability. However, results from the histological examination showed that visually inspecting the gonad was not a good indication of the actual level of gonad development. There is no evidence that the GI is a good indication of mature abalone eggs (Table 2). More recently, farmers have noticed that a large gonad does not necessarily mean that the abalone will spawn.

### Handling

Handling abalone did reduce egg production within the trial but this was not statistically significant. The nonhandled abalone produced approximately twice the average number of eggs than abalone that were handled regularly throughout the trial. This may not have been statistically significant because of the difference in the number of replicates used for the handled and non-handled groups of 2 tubs and 4 tubs respectively. Potentially, there may not have been enough power to detect a significant difference. Hone et al. (1997) found that in the wild abalone near condition will spawn if high stress conditions occur. Abalone farmers have suggested that handling stresses abalone.

There was no difference between the percentages of males and

females that spawned in each planned spawning induction. This does not follow the same pattern that was described by Hahn (1989), that in the hatchery when males and females are induced to spawn, a higher percentage of males spawn compared with females. However, our observation that males generally spawned before females confirmed that of Hahn (1989). In this trial, males usually spawned before females and commenced about 11–12 h after induction with UV. Initially, spawnings had finished ~17–18 h after UV induction. However, later observations showed that there were 2 "periods" of spawnings, the first being early to late evening (~11–18 h after UV induction) and the second being around dawn (~24 h after UV induction).

This system is suitable as a commercial conditioning system as it reduces labor costs in handling and provides less stress to the abalone before spawning. However, in comparison it would be less suitable for genetic programs based on single pair crosses because the abalone are held in groups and not removed from the tubs before spawning.

### ACKNOWLEDGMENTS

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## INFLUENCE OF CONDITIONING DIET AND SPAWNING FREQUENCY ON VARIATION IN EGG DIAMETER FOR GREENLIP ABALONE, *HALIOTIS LAEVIGATA*

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**ABSTRACT** Both nutrient provisioning of eggs, during development within the ovary, and egg diameter are believed to be important factors governing the development and survival of invertebrate larvae. The diet of female broodstock and the conditioning regimen are likely to influence these parameters. In this study we examine the effect of broodstock diet (three formulated diets differing in levels of the fatty acid, Arachidonic acid [ARA], and a red seaweed diet) and spawning frequency on egg diameter variability both within a batch of eggs spawned from one female and between batches of eggs. Greenlip abalone broodstock were spawned at the beginning of the experiment and again every 16 wks (1131 °C days effective accumulative temperature [EAT]) using commercial hatchery practices. The variability of egg diameter within batches spawned from the same female over three consecutive spawning rounds and within diet treatments was determined. Cytoplasm diameter, vitelline layer and jelly coat thickness of unfertilized eggs were also compared between eggs spawned from individual females and between females. In addition, the relationships between broodstock parameters (weight and shell length) and egg diameter were explored. Eggs spawned from females feeding on a red seaweed diet were smaller than eggs from the low ARA treatment. Depending on spawning frequency, broodstock diet can influence the cytoplasm diameter and jelly coat thickness. No relationship between egg diameter and broodstock parameters was found. However, batches spawned from the same female abalone became more variable over time with a shift in size frequency distribution. Results indicate that the variability of egg diameter within a batch changes from female to female and highlights the importance of selecting successful broodstock for conditioning at commercial hatcheries.

**KEY WORDS:** abalone, *Haliotis laevigata*, diets, egg diameter, arachidonic acid

### INTRODUCTION

One of the crucial factors controlling the development of the Australian abalone aquaculture industry is the successful conditioning of broodstock because the supply of suitable wild broodstock has at times been limiting. Gonad conditioning provides flexibility in production cycles and more economical use of hatchery resources (Leonart 1992) as well as allowing genetic improvement programs.

Abalone larvae are lecithotrophic and rely heavily on yolk reserves provided by the egg to fuel development (Jaekle & Manahan 1989). Previous studies have indicated that smaller eggs have improved fertilization rates and contain more total lipid than larger eggs (Daume & Ryan 2004). However, it is widely considered for invertebrates, that larger eggs produce larger offspring with a greater chance of survival (Leviton 2000), and that fertilization is increased at lower sperm concentrations for larger eggs (Leviton 1996, Marshall et al. 2002).

It has been proposed that the most appropriate method of determining reproductive cycles in molluscs and echinoderms is to measure oocytes (Grant & Tyler 1983). However, egg diameter, post spawning, is believed to be a key determinant of reproductive performance (Brooks et al. 1997) and small differences in egg diameter are considered to have significant biological consequences (George 1999). The ratio of cytoplasm to egg diameter is considered to be useful for assessing reproductive performance in blacklip abalone, *Haliotis rubra* Leach, (Littay & De Silva 2001) and to provide a measure of optimal egg size for fertilization experiments without the need for histological examination.

Earlier research on broodstock conditioning with greenlip abalone (*Haliotis laevigata*, Donovan) showed evidence of nutrient depletion of eggs during lengthy conditioning periods and high

spawning induction frequency (Daume & Ryan 2004, Freeman et al. submitted).

Thus nutrient provisioning of eggs and egg diameter are important factors governing the development and survival of larvae. The diet of female broodstock and the conditioning regimen are likely to contribute to the quality of abalone eggs.

A crucial step in successful abalone aquaculture is selecting female broodstock that exhibit high fecundity and produce high quality eggs over consecutive spawnings. This reduces the need to hold large groups of females and minimizes the amount of hatchery resources, such as feed, needed to maintain the broodstock.

This study examined the effect of diet (three formulated diets with differing levels of the fatty acid, ARA and a red seaweed diet) and spawning frequency on the variability in egg diameter within a batch of eggs spawned from one female and between batches and is part of a larger study of the influence of these diets on fecundity, egg quality and larval success.

### MATERIALS AND METHODS

#### Broodstock Conditioning

Greenlip abalone, *Haliotis laevigata*, were collected in August 2003 from Augusta (115°16'E; 34°32'S) and Hopetoun (120°13'E; 33°95'S), Western Australia and held indoors at Great Southern Marine Hatcheries, Albany, Western Australia.

Broodstock were randomly assigned to spawning groups (A to D), and within each spawning group there were 12 round 60 L plastic tubs of females and two tubs of males, with each tub containing 3 animals.

Broodstock were exposed to a photoperiod of 12L:12D at ca. 100 lux (measured at the bottom of tubs). The conditioning system received flow through, temperature controlled seawater (average temperature, 17 ± 0.2°C).

Broodstock (average length 132.5 ± 3.5 mm; average weight

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352.2 ± 27.6 g) were spawned prior to treatment conditioning and again every 16 wks. (i.e., every 1131 °C-days EAT). The study was conducted from October 2003 to October 2004 including a natural spawning season.

EAT was calculated using the formula (Kikuchi & Uki 1974):

$$Y_n = \sum_{i=1}^n (t_i - \theta)$$

where  $Y_n$  (°C days) = EAT,  $n$  (days) = number of days since water temperature rose above  $\theta$ ,  $t_i$  (°C) = daily water temperature in which the animal was reared, and  $\theta$  (°C) = biological zero point for gonad maturation (6.9°C for *H. laevis*; Grubert & Ritar 2003). Spawning dates were October 2003 for the spawn out, January/February 2004 for the first, May/June 2004 for the second and September 2004 for the third spawning (Table 1).

Within each spawning group (Table 1), 36 females (9 females per treatment) and 6 male greenlip abalone were individually induced to spawn using a combination of desiccation for 1 h, heat stress at 21°C and UV treated seawater.

#### Diet

Female broodstock were conditioned on four different diets, with three replicated tubs (9 animals in total) per diet treatment and per spawning group. One set of females were fed a formulated diet with a low enrichment level of arachidonic acid (EPA: ARA = 3:8; 1% of total fatty acids), the second set was fed a formulated diet containing a high level of ARA (EPA: ARA = 1:16; 2% of total fatty acids). A third set was fed a formulated diet containing no arachidonic acid (EPA: ARA = 6:0; 0% of total fatty acids). The formulation of the diet is proprietary. The fourth set of females was fed a mixed red seaweed diet (e.g., *Plocamium mertensii*, *Gracilaria* sp.). The mixed red seaweed was collected from the Southern Ocean mainly around Albany (117°95'E; 34° 90'S); the *Gracilaria* sp. was collected from Hopetoun (120° 13'E; 33° 95'S). Animals were cleaned and fed *ad libitum* daily. Feed intake was monitored daily.

#### Egg Measurements

Unfertilized egg samples were collected within 30 min of the female spawning and immediately fixed in 10% formalin. Thirty eggs were then measured using an eyepiece graticule on an inverted microscope. Cytoplasm diameter, vitelline layer and jelly coat thickness were compared between individual females, diet treatments and across consecutive spawnings (Fig. 1). The variability of egg diameter within batches spawned from the same female over three consecutive spawning rounds and within diet treatments were determined. Egg diameter includes the vitelline and cytoplasm components of the egg. The ratio of cytoplasm to egg diameter was calculated as the cytoplasm measurement di-

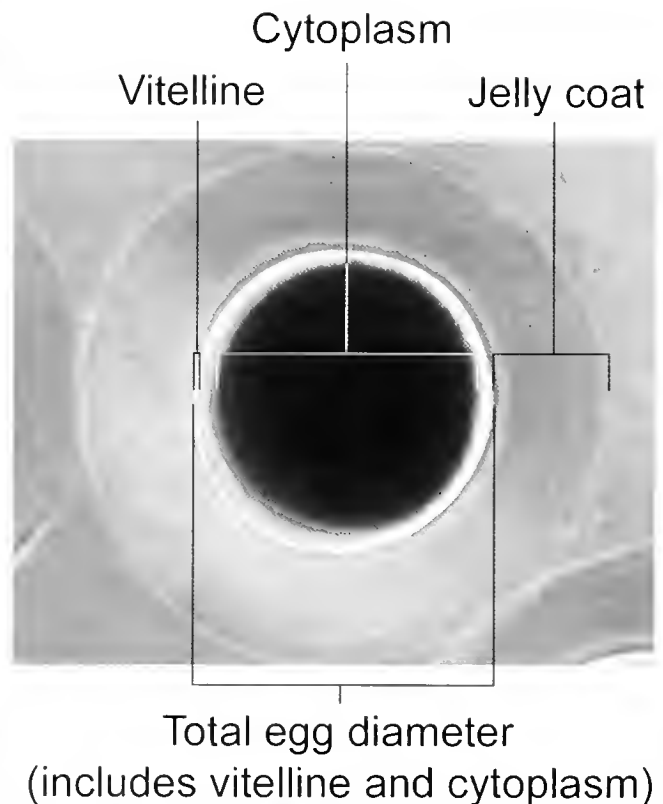


Figure 1. Position of cytoplasm, vitelline layer and the jelly coat in unfertilized eggs from *Haliotis laevis*. Total egg diameter includes the cytoplasm and vitelline layer.

vided by the egg diameter. Eggs were classed as primary eggs when the jelly coat and vitelline layer was absent and the total egg diameter was on average 111.51 ± 10.54 µm.

#### Data Analysis

All data analyses were carried out using Statistica software (version 6.0, StatSoft, Inc. 2002). Normality of all data was checked graphically using boxplots and with the Kolmogorov-Smirnov test.

Comparisons of egg parameters (cytoplasm, vitelline layer and jelly coat thickness), and comparisons of broodstock parameters with batch and egg size, were carried out using analysis of variance (ANOVA) with Tukey *post hoc* comparisons. Repeated measure analyses could not be performed, because different individuals spawned successfully during each of the three attempted spawnings.

Weight and shell length data in relation to weight loss were investigated using repeated measures ANOVA and Tukey *post hoc* comparisons. The relationship between broodstock parameters (length and weight) and egg diameter were explored with a simple regression analysis ( $P < 0.05$ ).

Size-frequency distribution of egg diameter were compared over time and between treatments where appropriate using descriptive statistics (minimum, maximum, size range, first and third quartile). Interquartile differences between the first and third quartile were used as a measure of spread of the size-frequency distributions.

TABLE 1.  
Spawning dates for each spawning group.

Spawning events	Spawning groups			
	A	B	C	D
Spawn out	07.10.03	15.10.03	22.10.03	29.10.03
First spawning	26.01.04	03.02.04	11.02.04	18.02.04
Second spawning	19.05.04	23.05.04	31.05.04	08.06.04
Third spawning	08.09.04	14.09.04	22.09.04	29.09.04

TABLE 2.

Influence of diet on cytoplasm diameter, vitelline layer and jelly coat thickness of abalone, *Haliotis laevigata*, eggs and the presence of primary eggs over three consecutive spawning periods (1, 2 and 3). (Data are means ( $\mu\text{m}$ )  $\pm$  SE)

Diet	Spawning	Cytoplasm	Vitelline	Jelly coat	Egg diameter ( $\mu\text{m}$ )	Ratio of cytoplasm to egg diameter	Percent occurrence of primary eggs (%)
Red seaweed	1	194.39 $\pm$ 1.52 <sup>a</sup>	18.17 $\pm$ 0.90	117.53 $\pm$ 3.89 <sup>b</sup>	212.52 $\pm$ 0.90	0.92	0.18 $\pm$ 0.13
	2	202.57 $\pm$ 1.02	22.10 $\pm$ 0.57	113.40 $\pm$ 1.37	219.67 $\pm$ 1.00	0.92	1.39 $\pm$ 1.39
	3	202.18 $\pm$ 2.14	24.47 $\pm$ 0.73	128.80 $\pm$ 7.77	224.49 $\pm$ 2.27	0.90	2.10 $\pm$ 0.90
High ARA	1	196.73 $\pm$ 1.55 <sup>ab</sup>	18.34 $\pm$ 1.37	103.92 $\pm$ 3.78 <sup>cd</sup>	215.06 $\pm$ 1.36	0.92	3.63 $\pm$ 1.23
	2	201.98 $\pm$ 1.31	27.28 $\pm$ 1.54	123.51 $\pm$ 3.16	224.88 $\pm$ 2.03	0.90	0.53 $\pm$ 0.53
	3	200.34 $\pm$ 1.24	26.08 $\pm$ 0.87	118.08 $\pm$ 4.25	226.61 $\pm$ 1.77	0.88	2.55 $\pm$ 1.30
Low ARA	1	204.34 $\pm$ 1.81 <sup>b</sup>	18.50 $\pm$ 1.27	92.39 $\pm$ 4.30 <sup>d</sup>	218.52 $\pm$ 1.27	0.94	5.55 $\pm$ 2.88
	2	207.24 $\pm$ 1.08	18.11 $\pm$ 0.76	115.10 $\pm$ 1.35	231.28 $\pm$ 1.55	0.90	5.65 $\pm$ 3.10
	3	201.25 $\pm$ 1.96	26.71 $\pm$ 0.93	117.44 $\pm$ 4.58	227.96 $\pm$ 1.52	0.88	1.49 $\pm$ 0.65
Control	1	196.53 $\pm$ 1.50 <sup>ab</sup>	17.44 $\pm$ 1.23	103.68 $\pm$ 3.84 <sup>cd</sup>	213.98 $\pm$ 1.23	0.92	3.90 $\pm$ 1.90
	2	202.36 $\pm$ 0.79	21.52 $\pm$ 0.66	121.26 $\pm$ 0.96	222.27 $\pm$ 1.29	0.91	3.98 $\pm$ 2.36
	3	203.38 $\pm$ 1.76	24.15 $\pm$ 0.86	117.73 $\pm$ 9.70	227.10 $\pm$ 2.05	0.90	3.05 $\pm$ 1.25

\* Means, within a column, with different superscript letters are significantly different ( $p < 0.05$ ).

## RESULTS

### Influence of Diets on Cytoplasm Diameter, Vitelline Layer and Jelly Coat Thickness, Egg Diameter and the Ratio of Cytoplasm to Egg Diameter of Abalone Eggs over Three Consecutive Spawning

During the first spawning, diet had a significant effect on cytoplasm diameter and jelly coat thickness ( $\text{df} = 9$ ,  $F = 2.13$ ,  $P = 0.03$ ), however there was no significant difference in vitelline thickness between treatments (Table 2). Eggs obtained from females fed the red seaweed diet were smaller in cytoplasm diameter than the low ARA treatment ( $\text{post hoc} = 0.005$ ). Jelly coat diameter was largest in the red seaweed treatment and significantly larger than in the low ARA treatment ( $\text{post hoc} = 0.007$ ).

Diet treatments were found to have no significant effect on cytoplasm diameter, vitelline layer or jelly coat thickness of eggs obtained from the second ( $\text{df} = 9$ ,  $F = 1.102$ ,  $P = 0.38$ ) and third consecutive spawning ( $\text{df} = 9$ ,  $F = 1.12$ ,  $P = 0.35$ ).

Across all spawnings, diet significantly influenced egg diameter ( $\text{df} = 3$ ,  $F = 3.24$ ,  $P = 0.023$ ). Egg diameter in the red seaweed diet was significantly smaller than egg diameter in the low ARA ( $\text{post hoc} = 0.015$ ), but did not differ significantly from the high ARA or control diet.

In all four diet treatments, the ratio of cytoplasm to egg diameter decreased between the first and third spawning rounds. The ratio was highest in the low ARA treatment during the first spawning round and decreased to 0.88 in the third spawning round. Similarly the ratio in the high ARA treatment decreased to 0.88 in the third round, from 0.92 in the first round and 0.90 in the second round.

The occurrence of primary eggs in batches spawned by females fed the red seaweed diet increased over the three consecutive spawning rounds (Table 2). However, primary eggs occurred more frequently in the three formulated diet treatments, particularly during the first spawning round.

### Relationship Between Diet, Shell Length, Weight and Weight Change of Female Broodstock over Three Consecutive Spawning Rounds

Table 3 shows the shell length, weights and weight changes of all female broodstock at each individual spawning including the

initial spawn out. Over the whole experimental period, all animals lost weight and time had a significant effect on weight loss ( $\text{df} = 3$ ,  $F = 11.78$ ,  $P = 0.00$ ). Broodstock weight at spawnout was significantly higher than weight during the first ( $\text{post hoc} P < 0.001$ ), second ( $\text{post hoc} = 0.000133$ ) and third ( $\text{post hoc} P < 0.001$ ) spawnings.

### Size Frequency Distribution of Eggs Spawned by the Same Female over three Consecutive Spawning Rounds

#### Red Seaweed

Eggs spawned by female one during the first spawning ranged between 208–229  $\mu\text{m}$ , with 75% (3rd quartile) of the eggs being within 210 and 224  $\mu\text{m}$  in diameter (Table 4, Fig. 2a). The spread

TABLE 3.

Influence of diet and spawning round on shell length, weight and weight change of *Haliotis laevigata*. (Data are means  $\pm$  SE)

Diet	Spawning round	Shell length (mm)	Weight (g)	Weight change (g)
Red seaweed	spawn out	130.28 $\pm$ 1.31	336.87 $\pm$ 12.00 <sup>a</sup>	
	1	129.78 $\pm$ 1.27	328.50 $\pm$ 10.74 <sup>b</sup>	-8.36
	2	129.39 $\pm$ 1.25	323.21 $\pm$ 10.95 <sup>b</sup>	-5.29
	3	129.89 $\pm$ 1.23	323.03 $\pm$ 9.97 <sup>b</sup>	-0.18
High ARA	spawn out	133.89 $\pm$ 1.37	380.24 $\pm$ 16.11 <sup>a</sup>	
	1	133.44 $\pm$ 1.35	363.47 $\pm$ 16.24 <sup>b</sup>	-16.77
	2	133.47 $\pm$ 1.44	360.98 $\pm$ 16.19 <sup>b</sup>	-2.50
	3	133.69 $\pm$ 1.45	352.57 $\pm$ 14.98 <sup>b</sup>	-8.41
Low ARA	spawn out	132.39 $\pm$ 1.40	352.26 $\pm$ 13.75 <sup>a</sup>	
	1	131.97 $\pm$ 1.39	330.18 $\pm$ 14.94 <sup>b</sup>	-22.09
	2	131.58 $\pm$ 1.38	331.53 $\pm$ 14.65 <sup>b</sup>	+1.35
	3	131.62 $\pm$ 1.52	327.36 $\pm$ 14.69 <sup>b</sup>	-4.17
Control	spawn out	133.33 $\pm$ 1.28	360.77 $\pm$ 11.92 <sup>a</sup>	
	1	133.31 $\pm$ 1.10	348.08 $\pm$ 10.03 <sup>b</sup>	-12.69
	2	133.17 $\pm$ 1.14	355.55 $\pm$ 10.73 <sup>b</sup>	+7.46
	3	133.63 $\pm$ 1.15	350.53 $\pm$ 10.61 <sup>b</sup>	-5.02

\* Means, within a column, with different superscript letters are significantly different ( $p < 0.05$ ).

of data became slightly more variable during the second spawning increasing to an interquartile difference of 7.23 from 5.26 during the first and third spawning. For the second female, variability in egg size decreased from the first to the second spawning and then increased slightly during the third spawning (Interquartile difference 7.23, 2.63 and 5.92 respectively). The spread of data for the third female was more consistent over all three spawnings with the interquartile difference remaining at 5.26. Egg diameter ranged between 210–230  $\mu\text{m}$ .

#### High ARA

Eggs obtained from this female fed the high ARA formulated feed were less variable over time, with eggs ranging between 203–221  $\mu\text{m}$  during the first spawning and between 210–230  $\mu\text{m}$  and 210 and 229  $\mu\text{m}$  in the second and third spawnings, respectively (Table 4, Fig. 2b). The interquartile difference stayed constant at 5.26 during all three spawnings.

#### Low ARA

The size frequency distribution of eggs obtained from the first female were highly variable between the first and second spawnings, with the distribution shifting from between 200–253  $\mu\text{m}$ , respectively (Table 4, Fig. 2c). The interquartile difference increased from 2.63–15.78 from the first to the second spawning and decreased to 6.57 during the third spawning. The second female only spawned during the first and second spawning, however the size variability was smaller and ranged from 210–239  $\mu\text{m}$ .

#### Control Diet (No ARA)

During the first spawning round eggs obtained from the first female ranged between 204–228  $\mu\text{m}$  with 75% (3rd quartile) of the eggs within 205 and 218  $\mu\text{m}$  in diameter (Table 4, Fig. 2d). The interquartile difference decreased slightly from 6.25–5.26 during the second spawning round, and was highest at 11.84 during the third spawning round. The eggs spawned by the second female were less variable during the first and third spawning. The interquartile difference was lowest during the first spawning (5.59) and increased to 7.89 and 7.56 during the second and third spawning respectively. The third female spawned during only the first and second spawning however, egg size decreased over these time periods with eggs ranging from 210–224 over the first and second rounds, respectively.

### DISCUSSION

#### Influence of Diets on Broodstock Parameters and Egg Parameters

The results of this study suggest that diet is not the only factor controlling the size of the eggs produced. Over all three consecutive spawnings, egg diameter in the red seaweed treatment differed significantly from only the low ARA diet, and relative differences varied with spawning rounds. During the first spawning round, eggs spawned by females fed the red seaweed diet were significantly smaller in cytoplasm diameter and had significantly larger jelly coats than those in the low ARA treatment. However, during the second and third consecutive spawnings there was no significant effect of diet on cytoplasm diameter, vitelline layer thickness or jelly coat thickness. Previous studies have indicated that diet directly influences egg quality in marine invertebrates (Jaekle 1995). On the other hand, Nevejan et al. (2003) found that the total lipid content of eggs and the size of the eggs spawned by the

TABLE 4.

Descriptive statistics of egg size frequency distributions spawned by 1–3 female abalone, *Haliotis laevigata*, per diet treatment over three consecutive spawning rounds.

Treatment	Spawning	Quartile	Female		
			1	2	3
Red seaweed	1	1. Quartile	218.29	213.69	213.03
		3. Quartile	223.55	220.92	218.29
		Interquartile	5.26	7.23	5.26
		Min (μm)	207.77	210.40	210.40
		Max (μm)	228.81	223.55	228.81
	2	1. Quartile	213.69	215.66	218.29
		3. Quartile	220.92	218.29	223.55
		Interquartile	7.23	2.63	5.26
		Min (μm)	205.14	210.40	210.40
		Max (μm)	223.55	230.13	226.18
	3	1. Quartile	218.29	213.36	218.29
		3. Quartile	223.55	219.28	223.55
		Interquartile	5.26	5.92	5.26
		Min (μm)	210.4	210.40	210.40
		Max (μm)	231.44	231.44	230.13
High ARA	1	1. Quartile	210.40		
		3. Quartile	215.66		
		Interquartile	5.26		
		Min (μm)	202.51		
		Max (μm)	220.92		
	2	1. Quartile	213.03		
		3. Quartile	218.29		
		Interquartile	5.26		
		Min (μm)	210.40		
		Max (μm)	230.13		
	3	1. Quartile	218.29		
		3. Quartile	223.55		
		Interquartile	5.26		
		Min (μm)	210.40		
		Max (μm)	228.81		
Low ARA	1	1. Quartile	207.77	221.58	
		3. Quartile	210.40	228.81	
		Interquartile	2.63	7.23	
		Min (μm)	199.88	210.40	
		Max (μm)	218.29	236.70	
	2	1. Quartile	220.92	223.55	
		3. Quartile	236.70	231.44	
		Interquartile	15.78	7.89	
		Min (μm)	202.51	218.29	
		Max (μm)	252.48	239.33	
	3	1. Quartile	216.98		
		3. Quartile	223.55		
		Interquartile	6.57		
		Min (μm)	210.4		
		Max (μm)	236.7		
Control	1	1. Quartile	212.04	202.18	213.03
		3. Quartile	218.29	207.77	220.59
		Interquartile	6.25	5.59	7.56
		Min (μm)	203.83	194.62	210.40
		Max (μm)	227.50	216.98	226.18
	2	1. Quartile	213.03	220.92	210.40
		3. Quartile	218.29	228.81	215.66
		Interquartile	5.26	7.89	5.26
		Min (μm)	205.14	194.62	202.51
		Max (μm)	226.18	244.59	223.55
	3	1. Quartile	211.72	216.98	
		3. Quartile	223.55	224.54	
		Interquartile	11.84	7.56	
		Min (μm)	186.73	193.31	
		Max (μm)	243.28	240.65	

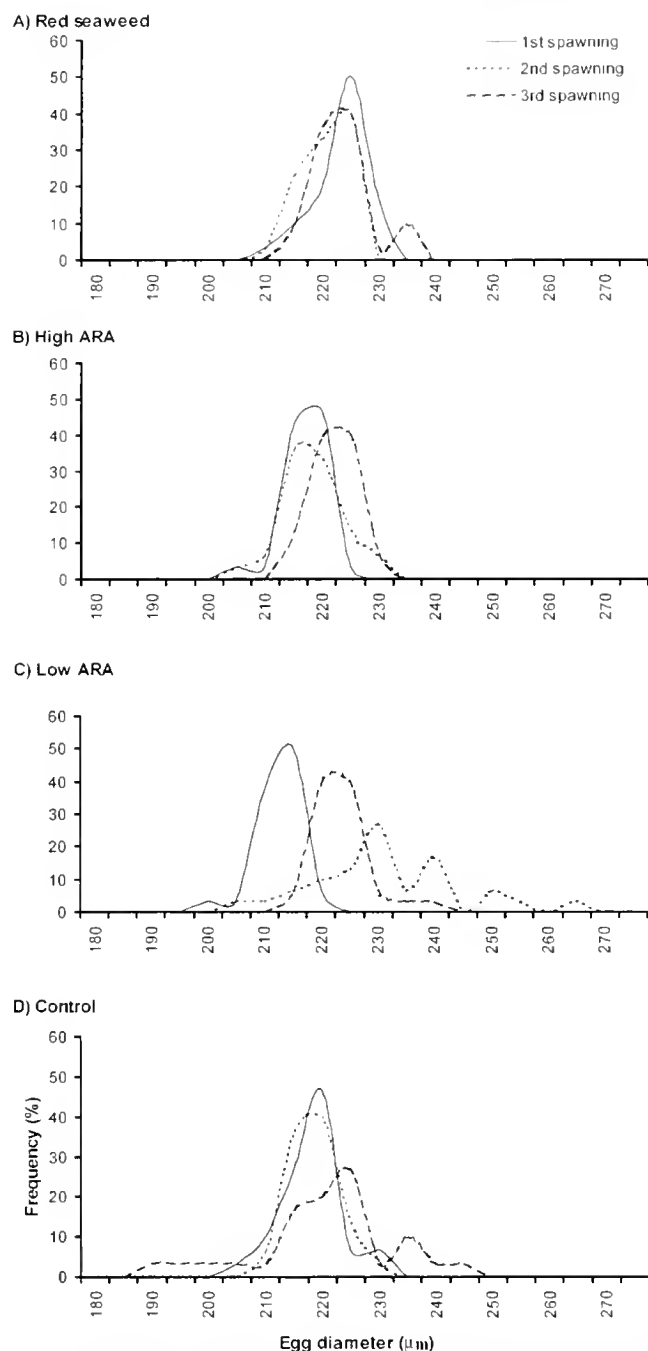


Figure 2. Size frequency distribution of eggs spawned by one female abalone, *Haliotis laevis*, over three spawning rounds for each treatment, (A) Red seaweed, (B) High ARA, (C) Low ARA, (D) Control.

scallop, *Argopecten purpuratus* were independent of diet. Similarly Caers et al. (2002) found there to be no significant effect of diet on the egg size of the oyster, *Crassostrea gigas*.

The role of diet in this study is unclear. Whether the increase in egg diameter in the low ARA treatment is correlated with the ARA content of the eggs will be determined after subsequent biochemical analysis of the eggs. It is likely however that the maternal condition of the female broodstock during the second spawning was negatively influenced by some other factor like overall animal nutritional condition. All animals lost weight during the experiment particularly when feeding on the formulated diet at the start of the experiment. It could be suggested that animals needed a

weaning period to get used to the new diets because weight loss was less apparent on the natural diet and decreased on the formulated diets after the first spawning.

If measurements of egg diameter are an accurate representation of egg ripeness, because larger eggs are more ripe than smaller eggs, then those eggs obtained from the low ARA formulated feed treatments were more ripe than those obtained from the red seaweed treatment. On the other hand the low ARA diet showed the highest percentage occurrence of primary eggs. It is possible that these larger eggs contained more moisture as found by Daume and Ryan (2004) and were thus not a true reflection of ripeness. It may also be that the micronutrient content of the formulated feeds may be lower than optimal. The red seaweed diet is a mixed species diet and subsequently may be lacking in some macronutrients. However, eggs in the red seaweed treatment showed the lowest percentage occurrence of primary eggs. A follow up study investigating combined broodstock conditioning diets, where broodstock are first fed a low ARA formulated diet and then finished off with red seaweed before spawning, is planned.

The decrease in the ratio of cytoplasm to egg diameter across all treatments may suggest that egg quality deteriorated over the consecutive spawnings. Littay and De Silva (2001) reported for blacklip abalone, that a ratio of yolk (equivalent to cytoplasm in this study) to total egg diameter between 0.83–0.87 was ideal for this species and provided the highest fertilization rate. These authors suggested that any deviation from this range (0.83–0.87) indicates overripe eggs or declining egg biochemical status. The ratios found in our study were much higher than those found by Littay and De Silva (2001) however that might be attributable to the different species investigated in this study.

#### Size Frequency Distribution of Eggs

The descriptive statistics of the size frequency distributions of eggs spawned from individual females suggest that the eggs within the gonad are not uniform in size and display high variability between consecutive spawnings. This may be directly related to conditioning time, with the broodstock needing a longer conditioning period to produce eggs of similar size and quality.

Huchette et al. (2004) found that development of blacklip abalone oocytes within the gonad was not uniform and resulted in eggs that were variable in size. Clavier (1992) suggested that only the largest and ripest oocytes within the gonad are released during a spawning. However, in this study egg size was very variable over single spawnings for some females. Broodstock may have responded to nutritional stress, resulting in resorption of ripe gametes. Martinez et al. (1992) concluded that a reduction in the gonad index of a hatchery-reared scallop, *Argopecten purpuratus*, was caused by resorption of ripe gametes because the broodstock were under nutritional stress.

In this study, egg diameter was measured as total egg diameter including the vitelline layer and the cytoplasm. However, according to Grant and Tyler (1983), the most appropriate method of determining reproductive cycles in molluscs and echinoderms is to measure oocytes. On the other hand, egg diameter is believed to be a key determinant of reproductive performance in fish (Brooks et al. 1997) and small differences in egg diameter are considered to have significant biological consequences (George 1999). Measuring egg diameter of unfertilized eggs (post spawning) is a relatively quick and simple means of gaining reproductive information about broodstock without the need of culling broodstock to measure oocytes.

## CONCLUSION

In this study we showed that the egg diameter varied between eggs spawned from females (*H. laevigata*) feeding on a red seaweed compared with the low ARA diet. Depending on spawning frequency, broodstock diet can also influence the cytoplasm diameter and jelly coat thickness. Egg parameter measurements and ratios of cytoplasm to egg diameter may be useful parameters for assessing reproductive performance in greenlip abalone. Studies are needed to further investigate the influence of broodstock diets on egg variability and ultimately on larval survival. Combination diets, where broodstock are fed a formulated feed for the better

part of the conditioning period, and then a suitable red seaweed species will be tested.

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## GROWTH, MORTALITY, RECRUITMENT AND SEX-RATIO IN WILD STOCKS OF SILVER-LIPPED PEARL OYSTER *PINCTADA MAXIMA* (JAMESON) (MOLLUSCA: PTERIIDAE), IN WESTERN AUSTRALIA

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**ABSTRACT** Growth, mortality, recruitment and sex-ratio of wild stocks of the silver-lipped pearl oyster *Pinctada maxima* were studied at sites spanning the geographic extent of the commercial fishery using mark-recapture experiments, recruitment cohort analysis and research surveys of stock abundance and reproductive status. Growth parameters ( $L_{\infty}$ , K) from the von Bertalanffy growth equation were estimated at 210 mm dorso-ventral measurement (DVM) ( $\pm 16$  mm SD) and 0.74 at the Lacepede Islands,  $L_{\infty}$  of 199 mm DVM ( $\pm 6$  mm SD) and K of 0.79 on 80 Mile Beach, and  $L_{\infty}$  of 194 mm ( $\pm 6$  mm SD), and K of 0.72 at Exmouth Gulf respectively. Estimates of natural mortality (M) by tagging were very low (0.02–0.03), compared with catch-curve analysis, which estimated M to be between 0.1 in deeper (30–34 m) populations and 0.18 in shallow (9–12 m) populations. Settled *P. maxima* spat (0 + and 1 + age classes) on adult shell were quantified (e.g., 1,317 spat found on 119,000 shell in 2003) to obtain an annual recruitment index, which showed clear temporal trends in abundance. Over 7 y (1992 to 1995; 2001 to 2003) the annual recruitment index varied from 5.1–8.0 spat per 1,000 shell for the 0 + age class, and 3.5–6.2 spat per 1,000 shell for the 1 + age class. Preliminary predictions of future abundance showed promise, however more work is required on spatial and habitat effects on spat settlement before the potential of the 0 + and 1 + recruitment indices can be realized. We also confirm that Western Australian populations of *P. maxima* are protandrous hermaphrodites, with a 50:50 sex ratio not achieved until females are 170 mm DVM, which is above the maximum size fished.

**KEY WORDS:** pearl oyster, *Pinctada maxima*, growth, mortality, recruitment, sex-ratio

### INTRODUCTION

Wild stocks of *Pinctada maxima* (Jameson) underpin an AU \$120 million dollar pearling industry in Western Australia and the fishery has been in operation for over 120 y. From the late 1880s up to the mid 1990s, the industry relied primarily on wild caught shell for pearl production. Since then, a concerted shift towards the culture of *P. maxima* led to the determination of the reproductive cycle of stocks (Rose et al. 1990), techniques for larval and spat culture (Rose & Baker 1994) and the assessment of growth and mortality in hatchery and nursery culture (Mills 2000, 1997, Taylor et al. 1997, Yukihira et al. 1998). This focus on the cultured animal is not surprising; with the exception of Western Australia, there are no substantial wild stocks left anywhere in the Indo-Pacific. Even the estimation of spat settlement and recruitment of wild stocks has been primarily directed towards the potential of farming wild-caught spat for pearl production (Beer & Southgate 2000, Knuckey 1995). Knuckey (1995) suggested that artificial collectors of natural spat could be used as a stock assessment tool, as is the case in scallop fisheries. However, early trials determined that logistical difficulties (12 m tidal ranges) associated with the deployment of artificial spat collectors precluded their satisfactory application in Western Australia (Joll 1994).

In the 1950s, Takemura and Okutani (1955, 1958) identified the “piggyback spat” phenomenon, whereby new recruits of *Pinctada* sp settled on the backs of adult oysters and remained attached for at least the first year or two of their life. Given that over half a million oysters are caught each year in the fishery, we hypothesized that “piggyback spat” sampled from the wild caught shell may provide a useful broad scale index of recruitment in this species. The rate of “piggyback” spat found on pearl oysters caught by the commercial fishery was examined for its potential as a method for monitoring and measuring recruitment to the pearl

oyster stock. This provides a basis for understanding the annual variation in recruitment and predicting the level of recruitment to the fishery 2–3 y later. The current recruitment index is based on oysters reaching legal minimum size (120 mm shell length) and can include a combination of year classes.

Despite the long-history of exploitation of this pearl shell, there have been no published estimates on key demographic parameters of wild stocks. The aims of this study are therefore, to provide comprehensive estimates of growth, mortality, recruitment and sex-ratio in commercially harvested wild stocks of *P. maxima*.

### MATERIALS AND METHODS

#### Growth

Growth was assessed with data from different stages of the life history; growth-increment measurements from tag-recapture experiments at the northern (Lacepede Islands), central (80 Mile Beach) and southern (Exmouth Gulf) areas of the commercial fishery (Fig. 1), and cohort analysis of the 0 + and 1 + age classes in successive years. Oysters for the tag-recapture experiment were obtained from commercial and research diving. Prior to measurement and tagging, oysters were held in plastic holding crates hung from the side of the boat or aerated seawater on deck ( $< 4$  h) or when available, in purpose built seawater through-flow tanks on pearling boats (up to 12 h). All releases and recaptures were made during a single neap (tide) in June of each year at the 80 Mile Beach site, during a single neap in February/March each year at the Lacepede Channel site and during a single neap in June/July each year at the Exmouth Gulf site. Overall, annual growth increments from 2,700 recaptured animals (Table 1), combined with modal length frequency of 0 + and 1 + cohorts over 8 years were used in the determination of growth of *P. maxima*.

Oysters were tagged using plastic shellfish tags (Hallprint, South Australia) measuring approximately  $15 \times 7$  mm ( $7 \times 3$  mm for smaller oysters  $< 50$  mm DVM). These tags were applied to the

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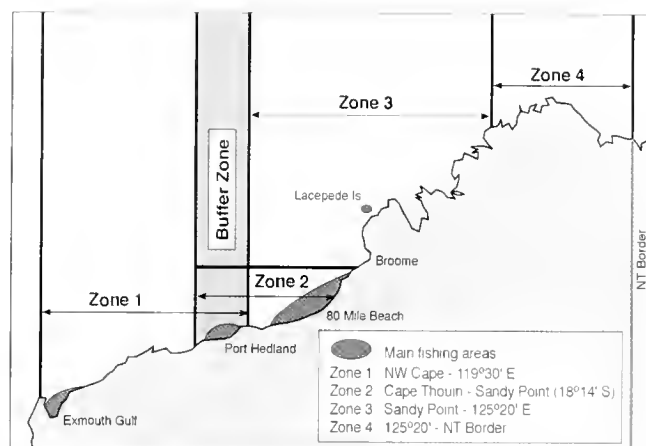


Figure 1. Management areas of the Western Australian *Pinctada maxima* fishery showing the three populations examined for growth (Exmouth Gulf, 80 Mile Beach, Lacepede Islands). The buffer zone is the area in which license holders in both Zone 1 and Zone 2 may harvest pearl shell.

left and right valves of each oyster using a cyano-acrylate glue (Selleys or Loctite 454 Gel). Each oyster was tagged using a pair of tags with the same 4 character identifier. The dorso-ventral measurement (DVM) of each oyster was recorded using standard calipers and measuring boards. Other morphometric measurements (anterior-posterior, hinge line, hinge depth and thickness) were taken, but only the DVM results are reported here.

Oysters were released on commercially fished habitat characteristic of each region. At the 80 Mile Beach site and Lacepede Channel, oysters were released in 15–18 m depth; at Exmouth Gulf oysters were released in 5–8 m. Oysters recaptured during research surveys were remeasured in successive years and rereleased in the same area. Any new pearl oysters found during recapture surveys were tagged, measured and released along with the recaptured oysters. During the period of growth monitoring, the pearling industry avoided fishing in the vicinity of tag sites.

Growth was analyzed using a maximum likelihood reformulation of the Von Bertalanffy growth curve for tagged data (Francis 1988). Equations 1 and 2, and Table 2 of Francis (1988) describe the reformulated equations and parameters. Model fitting is conditional on specifying two appropriate lengths described by the data ( $\alpha$  and  $\beta$ ), from which mean annual growth ( $g_{\alpha}$ ,  $g_{\beta}$ ) and  $K$  and  $L_{\infty}$  is calculated and the resultant fit examined. The best fit (maximum log-likelihood) for the *P. maxima* growth model occurred with the following values of  $\alpha$  and  $\beta$  for each region. Lacepede

TABLE 1.

Period at liberty (years) and number of recapture increments used to assess growth of *Pinctada maxima* in Western Australian populations.

Location	Year					
80 Mile Beach	Period at Liberty	89/90	90/91	91/92	92/93	93/94
	No. Recaptures	204	362	553	343	391
Lacepede Channel	Period at Liberty	96/97	97/98			
	No. Recaptures	293	84			
Exmouth Gulf	Period at Liberty	96/97	97/98			
	No. Recaptures	275	212			

TABLE 2.

Recruitment (piggyback spat) monitoring of *Pinctada maxima* in Western Australia, showing the number ( $n$ ) and percent (%) of oysters caught commercially that were examined for the presence of spat.

Year	Sampling Regime	Location 80 Mile Beach (Zone 2)	Lacepede Islands (Zone 3)
1991	$n$	17,867	
	$\%$	5%	
1992	$n$	20,950	15,536
	$\%$	6%	18%
1993	$n$	31,252	13,238
	$\%$	8%	19%
1994	$n$	72,284	15,008
	$\%$	19%	19%
1995	$n$	83,134	21,576
	$\%$	20%	24%
2001	$n$	132,920	5,020
	$\%$	28%	18%
2002	$n$	123,660	9,340
	$\%$	27%	53%
2003	$n$	107,670	11,608
	$\%$	25%	50%
2004	$n$	101,833	6,593
	$\%$	27%	26%

Islands (50, 170 mm DVM), 80 Mile Beach (60, 160 mm DVM) and Exmouth Gulf (65, 180 mm DVM). We also use the variability in growth-increment data to estimate the standard deviation of  $L_{\infty}$ , as described by Francis (1988).

#### Mortality

Pearl shell fishing in Western Australia is a gauntlet fishery targeting 3 age classes (3–6 y olds) in the 120–170 mm DVM size range, with larger animals unexploited. Natural mortality was determined directly and indirectly for the unexploited portion of the stock (shell >170 mm DVM) by tag and recapture studies conducted on fixed transect lines and by examining stock size-structure and undertaking length converted catch-curve analysis (Pauly 1984).

#### Mortality: Tagging Data

*Pinctada maxima* were placed in a predefined grid area and monitored at successive time intervals. Five parallel lines were established at 6-m depth in the Gales Bay area of Exmouth Gulf in July 1998. The lines were secured at the ends with anchors along a north-south direction approximately 10 m apart, and numbered 1–5 from east to west. Short lengths of chain were attached to the rope at regular intervals to assist in anchoring each line to the bottom. Oysters were collected from surrounding stocks, brought to the surface, placed in baskets suspended in the water from the side of the boat and tagged and measured as described in the growth section. The number of shell collected, processed and placed on the bottom each time was minimized (approximately 40–50), to ensure the pearl oysters were stressed as little as possible from the catch and tag procedure. Oysters were then hand placed on the substrate next to one of the five lines on the seabed.

A total of 721 oysters were tagged and released between July and August 1998. The size range tagged was 60 mm to 240 mm DVM, with over 75% of shell being greater than 170 mm.

To separate natural mortality from experimental mortality (oysters stressed by the collection and translocation to experimental sites), shell were allowed to recover for 1 mo after the initial tag and release. Experimental mortality was estimated for the 1-mo period, after which the estimation of natural mortality began. It was assumed stressed shell would either have died or recovered to a naturally healthy state after a month.

The intention was to collect mortality data after pearl oysters had been at liberty for 1 and 2 y (August 1999 and 2000). However, during the first assessment of the experiment (August 1999), the destructive effects of a category 5 cyclone (Vance, March, 22 1999), which produced Australia's highest ever recorded wind gust of 273 km/h, became apparent. Experimental lines suffered a similar fate to equipment on nearby commercial farms, where 395 mm of rainfall run-off, high winds, and storm surge had caused widespread movement of oysters and equipment. Overall survival rate of shell was quite high, as a large number of pearl oysters were found alive ( $n = 221$ , 48%), however there was no discernable structure left to the experimental layout and results obtained were not deemed representative of a normal year.

Consequently, a repeat experiment was set-up in Exmouth Gulf on the August 8, 1999 to determine survival of shell over a single year period. Seven clumps of 20 tagged shell spaced approx 0.8 m apart, were positioned by divers along four 50-m lines (Fig. 2). The experiment sites were revisited a year later when divers retrieved, recorded and measured the tagged oysters. This sampling layout simulated the natural distribution of shell on the bottom.

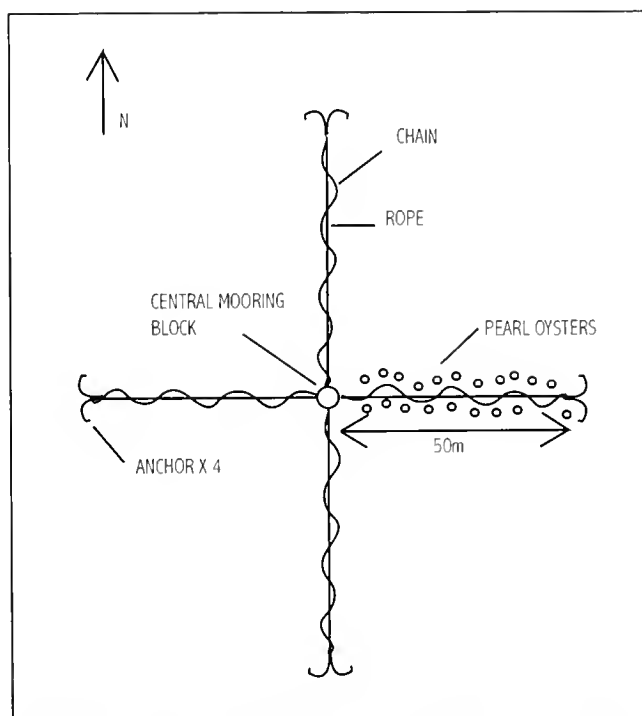


Figure 2. Design of mortality study site in Gales Bay, Exmouth.

#### Mortality: Length-converted Catch Curves

Length-converted catch curve analysis was used to estimate natural mortality in the unfished portion of the population above 170 mm. First, age-length keys were generated from the parameters of the von Bertalanffy growth equation, with variations of age at length obtained from the residuals in the tag-recapture model fitting (Haddon 2001). Second, shell lengths from research surveys of the northern (Lacepede Channel and Hama Patch) and 80 Mile Beach populations (5 locations: Patterson Shoal, 10 Mile, 13–15 Mile, 17 Mile, Compass Rose) were allocated to ages using proportions in the age length key. No model was run for oysters in Exmouth Gulf because there was no detailed survey of these stocks to provide length frequency information.

Natural mortality ( $M$ ) was assessed by determining the negative slope of the regression line from log frequency estimates of numbers at age for those shells above 170 mm (Pauly 1984). Mortality was estimated for 4 locations (Lacepede Islands, 80 Mile Beach Inshore, 80 Mile Beach Offshore, Compass Rose).

#### Recruitment

Research and industry personnel inspected oysters collected by pearl divers for the presence of settled *P. maxima* spat, which were separated into two age classes (Fig. 3). After each drift, between 100 and 300 shell were caught and placed in a central pile for cleaning. Twenty oysters at a time were randomly selected from the pile, each one inspected, spat collected and measured, and the process repeated until a representative subsection of the catch had been sampled. Between 18,000 (1991) and 137,000 (2001) adult pearl oysters were examined each year for the presence of spat (Table 2). Currently, this sampling effort evaluates 25% to 30% of oysters caught each year. A comparison of spat abundance between research and industry data was also undertaken.

#### Sex Ratio

Rose et al. (1990) and Hancock (1993) quantified the reproductive cycle of wild stocks of *P. maxima* in Western Australia, and our sampling utilized this knowledge to determine the population sex-ratio. To coincide with the beginning of the predicted spawning season, sex-ratio data were examined from oysters collected from 80 Mile Beach in October 1998 ( $n = 479$ ). DVM of all specimens was recorded and the valves of each oyster were partially opened to inspect gonad condition and determine gender by visual inspection of gonad color (males, white to cream colored gonad; females, pale yellow to orange colored gonad). Animals in which the gonad was a watery white color or appeared translucent were recorded as indeterminate. Sex-ratio variation by size was examined.

## RESULTS

#### Growth

*Pinctada maxima* obtains an average maximum theoretical length of between 193 and 210 mm DVM, and a von Bertalanffy growth coefficient ( $K$ ) of 0.72–0.79 (Table 3). Growth curves and residual analysis of model fits describing growth in *P. maxima* are shown in Figure 4. Oysters grow faster and attain a higher maximum size in the Lacepede Islands, in comparison with the 80 Mile Beach and Exmouth Gulf stocks.

A comparison of length frequency of the commercial catch and research surveys for both populations (Lacepede Islands

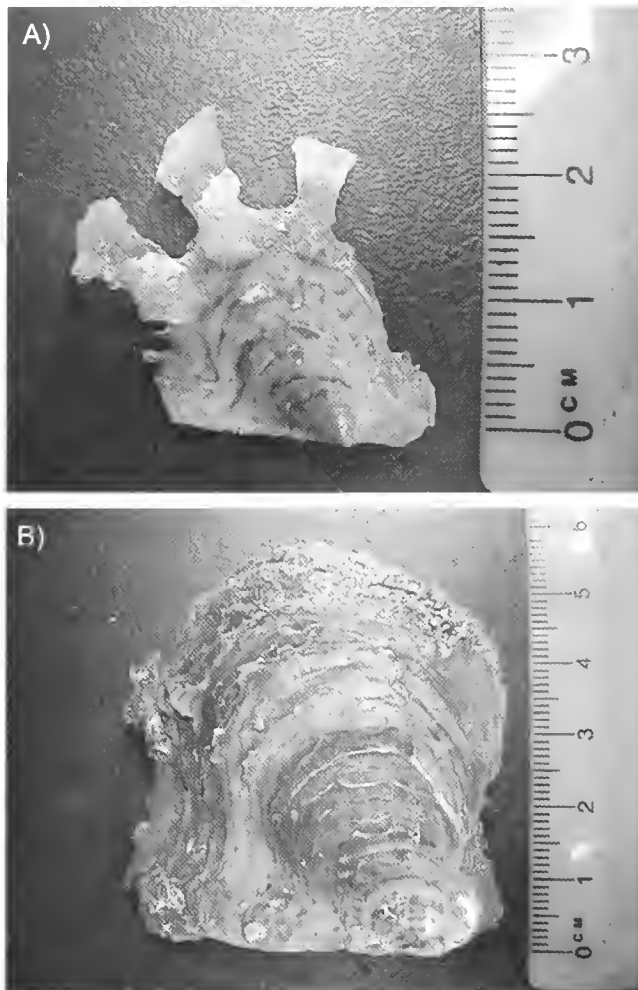


Figure 3. *Pinctada maxima* spat found on commercially fished adult shell. (A) O + spat, 17 mm DVM, approximately 4 mo old; (B) 1 + spat, 57 mm DVM, approximately 16–18 mo old.

and 80 Mile Beach) confirms the growth curve results, namely a larger size of *P. maxima* at the Lacepede Islands (Fig. 5).

Spat cohort data shows growth to be relatively slow in the first 18 mo, with average annual increment between 0+ and 1+ cohorts varying from 28–36 mm, the exception being 1995, which at 42 mm, appears to have been an exceptionally fast growth year (Fig. 6).

TABLE 3.

Growth parameters and their variability for the silver-lipped pearl oysters (*Pinctada maxima*) at three locations in Western Australia.

Growth Parameters <sup>1</sup>	Lacepede Islands	80 Mile Beach	Exmouth Gulf
$\alpha$ (mm)	50	60	65
$\beta$ (mm)	170	160	180
$g_{\alpha}$ (mm year <sup>-1</sup> )	42	30	37.5
$g_{\beta}$ (mm year <sup>-1</sup> )	10	8	9.5
$L_{\infty}$	210	200	194
Standard deviation of $L_{\infty}$	5.0	16.2	5.3
K	0.74	0.79	0.72

<sup>1</sup> The parameters are derived from the Francis (1988) reformulation of the von Bertalanffy growth curve. See methods for more details.

#### Mortality: Effect of Handling on Tag Retention

Of the 93 shell released in Exmouth Gulf in July 1998, 92 were found 1 mo later in August 1999. From these 92 shell, 2 were dead, 3 had damaged tags and 1 shell had lost both tags. Therefore, total mortality from experimental handling was  $2/92 = 2.17\%$ , and tag loss was 4.3%.

#### Mortality: Tagging Estimates

Of the 200 oysters placed-out for 1 y in August 1999, 195 were retrieved live in August 2000, 2 were dead and three were not relocated. The collection rate of 195/200 (97.5%) indicates that adult mortality was low (2.5% p.a.). Thus, overall direct estimates of annual natural mortality of adult pearl shell (6+ years) were 1% to 2%, which equates to M of 0.02.

#### Mortality: Catch Curve Analysis of Adult Pearl Shell (6+ years)

Natural mortality (M) ranged from 0.18 or 16.5% per year (80 Mile Beach, inshore shallow) to 0.1 (10%) at the Compass Rose deepwater stocks (Fig. 7). Mortality at the Lacepede Islands (0.148) and 80 Mile Beach offshore shallow was intermediate between these. This trend is correlated with depth (i.e., highest mortality in shallower waters and lowest in deep in the 80 Mile Beach stocks).

#### Recruitment

Separation of 0+ and 1+ age classes of *P. maxima* spat were obtained consistently over 9 y of sampling the 80 Mile Beach stocks (Fig. 6), and 0–34 mm spat are considered 0+ age (5–8 mo), with 35–75 mm considered 1+. This enabled clear temporal trends in spat settlement to be quantified (Fig. 8), particularly in the Lacepede Islands (Fig. 8). However, the magnitude of the recruitment index differed between research and industry data collection, particularly for the 0+ age class (Fig. 9). Despite this, there was still a significant positive correlation between industry and research personnel data for the 0+ age class ( $n = 4$ ;  $r = 0.95$ ;  $P < 0.05$ ). For the 1+ age class, there was also a positive correlation between industry and research personnel data ( $n = 4$ ;  $r = 0.67$ ;  $P > 0.05$ ), however it was not significant, mainly because of the small sample size (Fig. 8).

#### Predicting Future Stock Abundance with Recruitment Data

In Zone 2, there was a high positive correlation between 0+ spat abundance and 1+ spat abundance 1 y later for the industry data ( $n = 4$ ;  $r = 0.89$ ), and the research data ( $n = 3$ ;  $r = 0.92$ ). Neither was statistically significant because of the small sample sizes, however they are sufficiently high to enable reasonable confidence in their predictive potential, which needs to be confirmed with more years of sampling.

#### Sex Ratio

Sex-ratio data confirms that *P. maxima* is a protandrous hermaphrodite (Fig. 10). Size-at-maturity for males was around 110 mm, females were identified from 135 mm onwards and the sex ratio reached 50:50 female to male at approximately 170 mm DVM. From 170–200 mm there was a greater proportion of females in the population (Fig. 10).

#### DISCUSSION

Results of this study establish for the first time, the crucial demographic parameters of growth, mortality, recruitment and

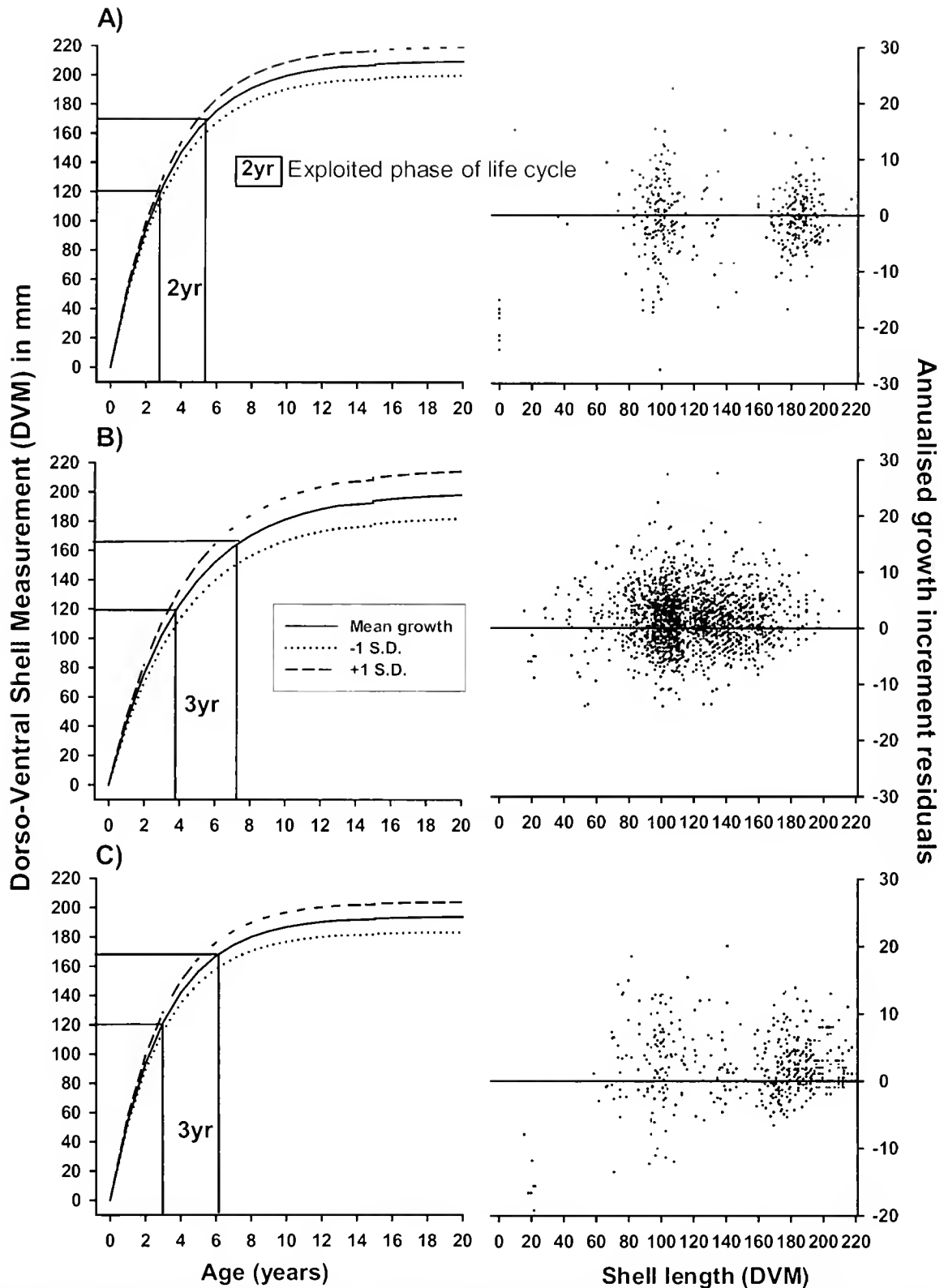


Figure 4. von Bertalanffy growth curves for each population of *Pinctada maxima* (A) Lacepede Islands; (B) 80 Mile Beach; (C) Exmouth Gulf) and annualized growth increment residuals. The exploited phase of the life cycle in each population is also shown.

sex-ratio of wild stocks of *P. maxima* in Western Australia. Growth was estimated *in-situ* for the 3 main commercially fished stocks, the Lacepede Islands, 80 Mile Beach, and Exmouth Gulf stocks, and represent the first published results for *in-situ* assess-

ment of growth of wild stocks of *P. maxima*. This is in contrast to farmed stocks of *P. maxima*, where there have been many assessments of growth, beginning with Wada (1953). Our growth results confirm that the wild fishery in Western Australia is a gauntlet

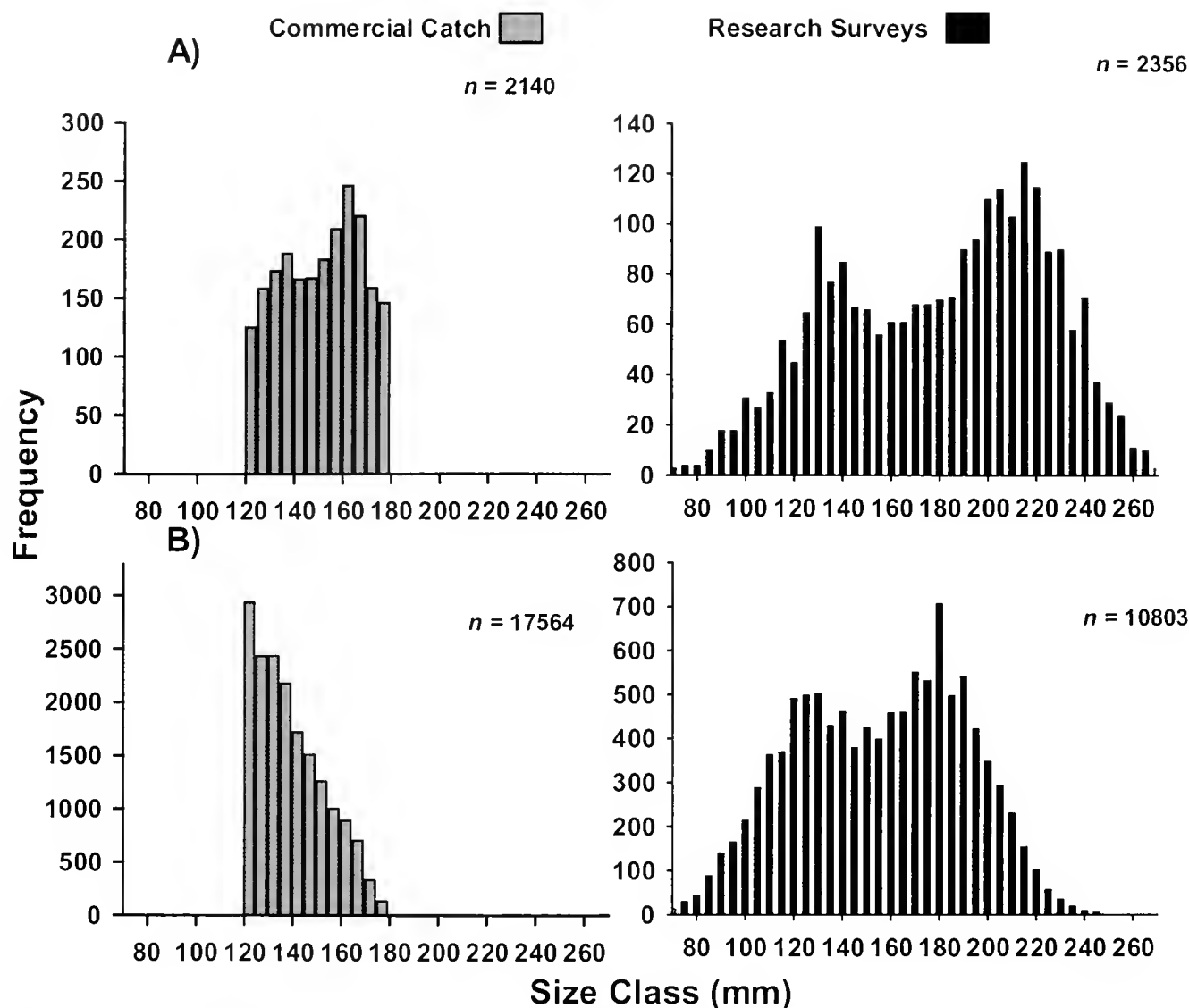


Figure 5. Size-frequency of *Pinctada maxima* found (A) Lacepede Islands (Zone 3) and (B) 80 Mile Beach (Zone 2). Commercial catch data and research survey data are both shown. The two modal peaks in the research survey data are not age classes but result from selective fishing.

fishery, targeting between 2 and 3 age classes, depending on location, and that average growth in the initial years from 0 + to 1 + is relatively slow, at around 30 mm per year, although it can vary considerably between years. A secondary spawning peak of *P. maxima* in March to April (Rose et al. 1990) may affect the integrity of the 1 + cohort data to some degree, however there is enough consistency between years in spat size frequency to be confident that 30 mm per year is an accurate average for 20–40 mm *P. maxima* spat on the 80 Mile Beach. This is slower than growth rates achieved by *P. maxima* spat grown near the surface on long lines, which generally achieve an average increment of 50 mm per year in their first 2 years at farms in the Northwest and North of Australia (D. Mills, pers. comm.). Cooler temperatures and surface boundary effects creating slower flow rates are hypothesized to be the main causes of slower growth in spat from 80 Mile beach stocks. Rose et al. (1990) recorded winter water temperatures dipping to 20°C, which is close to the calculated temperature of 0 growth (18°C) in *P. maxima* (Mills 2000, Pass et al. 1987). Also, spat were collected from wild adult oysters living in

benthic habitats where friction effects naturally slow the average water movement, in comparison with cultured spat that are positioned individually on surface long lines. This practice provides optimal, rather than normal, conditions for growth. Other studies of tropical bivalves also confirm the growth enhancing effect of surface long lines; for example Hart et al. (1999) showed that juvenile growth of a giant clam (*Tridacnidae*) (0–2 y) was considerably improved on surface long lines.

Rose et al. (1990) reported that males in the wild mature at 1 y with a shell height of 110 mm, however our growth results indicate this is incorrect. At 110 mm DVM, the size when males start to mature, their age would be 2 y, and most would be in their third year of life, at least on the 80 Mile Beach stocks. Females do not mature until 4 or 5 y of age at 140 mm DVM. These results show clearly, for the first time, that growth in wild stocks of *P. maxima* is less than that achieved on farms.

In Western Australia, unfished shells grow into the "Mother-of-Pearl" or MOP size-class at 170 mm DVM + and remain unexploited at a low natural mortality rate. Estimates of the age of an

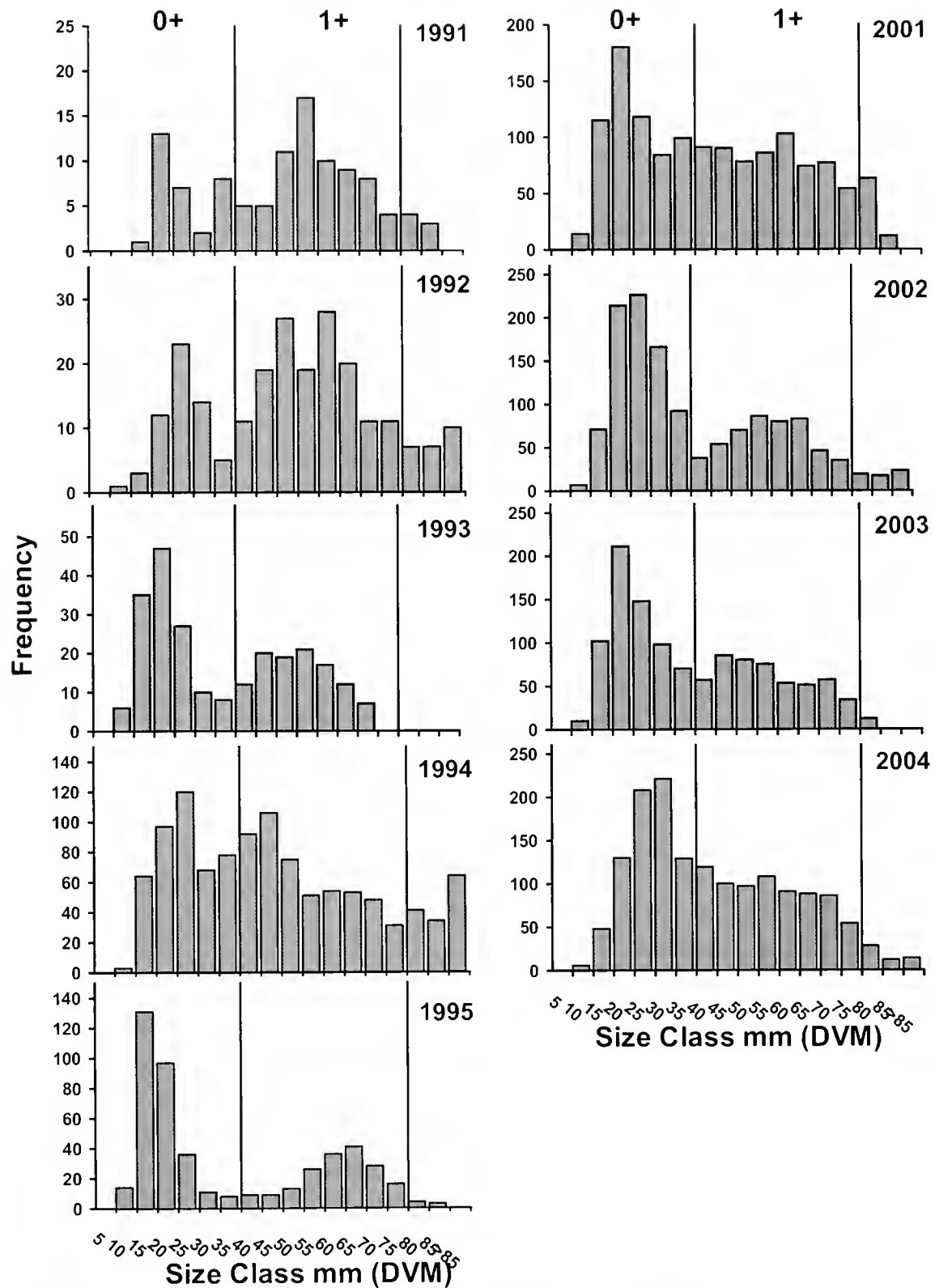


Figure 6. Size frequency of 0 + and 1+ *Pinctada maxima* spat collected from recruitment surveys during the period 1991 to 2004.

exceptionally large oyster found on the 80 Mile Beach fishing grounds (252 mm DVM) are between 15 and 30 y (Hart, unpublished data), hence it is possible that *P. maxima*, which escape commercial exploitation may breed for another 10–15 y. Overall,

Research surveys of MOP have confirmed high abundance of stocks (Hart & Friedman 2004) and preliminary analyses suggest that the balance between recruitment and fishing mortality is generally tipped towards recruitment, except in exceptionally poor

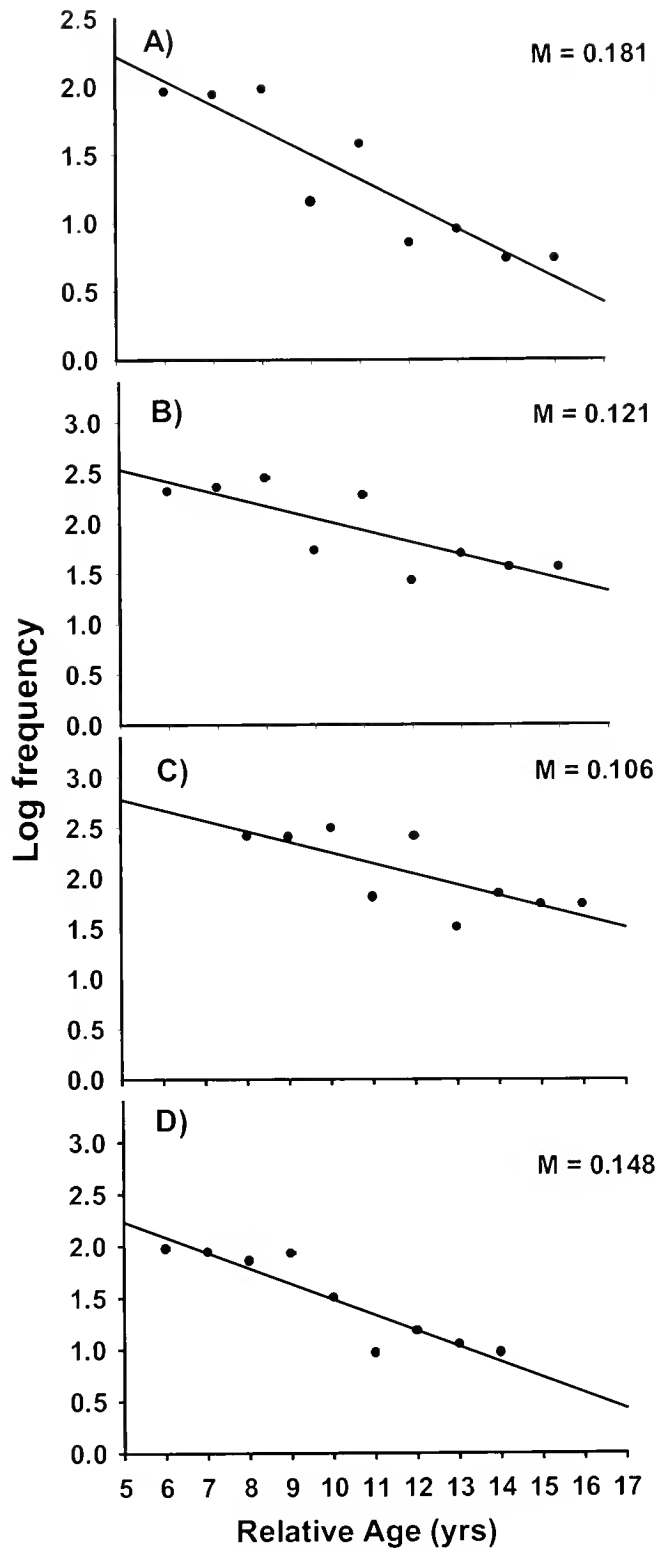


Figure 7. Length converted catch curve analysis for *Pinctada maxima* sampled at 4 populations. (A) 80 Mile inshore shallow; (B) 80 Mile - Offshore shallow; (C) Compass Rose; (D) Lacepede Islands.  $M$  = natural mortality.

recruitment years. Stock surveys however, should be carried out every 5 y to measure any change of the breeding stock.

Comparative estimates of the demographic parameters  $K$  and  $L_{\infty}$  for pearl oysters are sparse in literature, owing presumably to

the fact that pearl fisheries were among the earliest marine animals exploited commercially, most of which occurred prior to the development of modern, quantitative biological methods. Saucedo et al. (1998) for example, reports that the natural pearl beds of *Pinctada mazatlanica* in Mexico were subject to 400 y of uncontrolled exploitation, leading to virtual extinction by 1939. Herdman (1903) reports on the great antiquity of the Ceylon pearl oyster fishery, which targeted *Pinctada imbricata* and details individual yearly catches and revenues of this fishery throughout the 1800s at a very fine spatial scale, including an assessment of normal and stunted growth in the presence of overcrowding, however this was before von Bertalanffy's (1938) seminal work. Sims (1992) study on the black-lip pearl oyster (*Pinctada margaritifera*) in the Cook Islands yielded an  $L_{\infty}$  infinity of 183 mm, and  $K$  of 0.26, which is slightly smaller and slower growing than *P. maxima*. This is to be expected because *P. maxima* is recognized as the largest of all pearl oysters in the world (Shirai 1994), although Saucedo et al. (1998) presented data on individual *P. mazatlanica* growing to 190 mm DVM. Considerable spatial and individual variation in growth of *P. maxima* in Western Australia was noted, with a standard deviation of 16 mm from the Zone 2 stocks, and exceptional individuals have been observed as large as 300 mm from the Lacepede Islands. This is confirmed by Wada (1953), who reports that a 300 mm specimen was not uncommon in some fishing areas along the north of Australia and Arafura Sea.

Natural mortality ( $M$ ) of 170 mm + oysters appeared to be

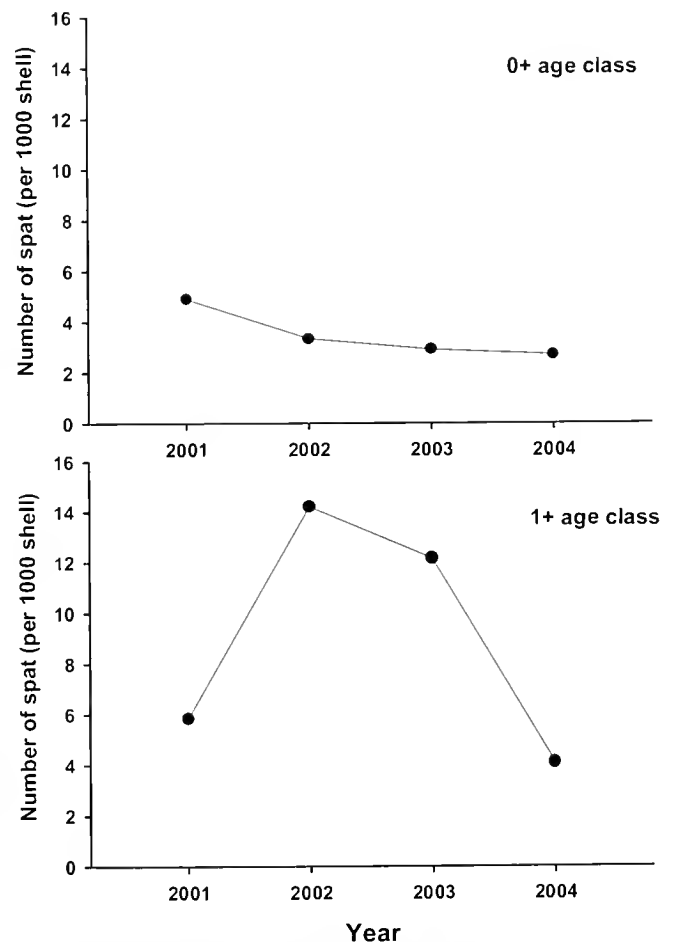


Figure 8. Trends in abundance of 0+ and 1+ spat of *Pinctada maxima* in Zone 3 (Lacepede Islands) from 2001-2004.



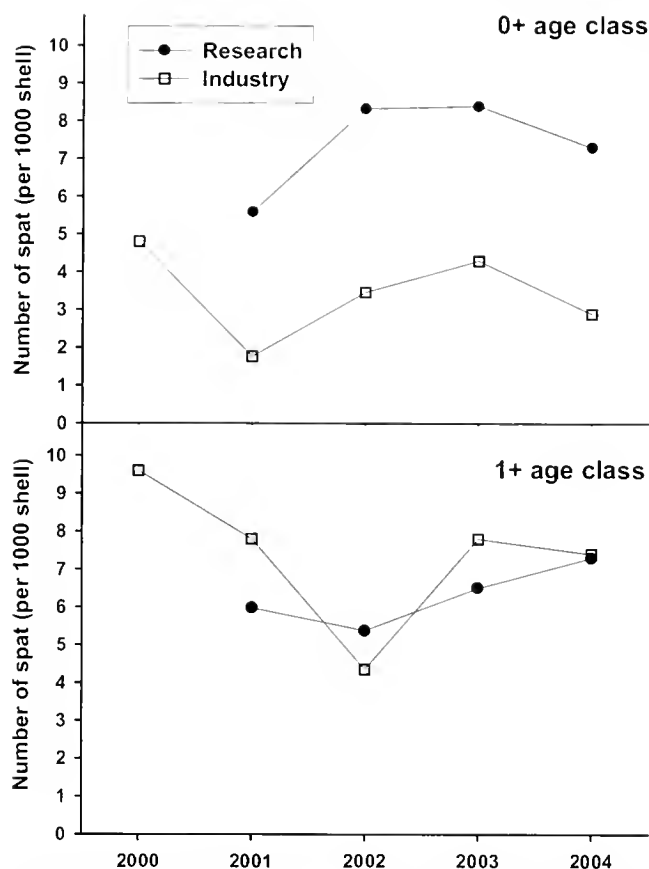


Figure 9. Trends in abundance of 0+ and 1+ spat of *Pinctada maxima* in Zone 2 from 2001 to 2004 (Research and industry data).

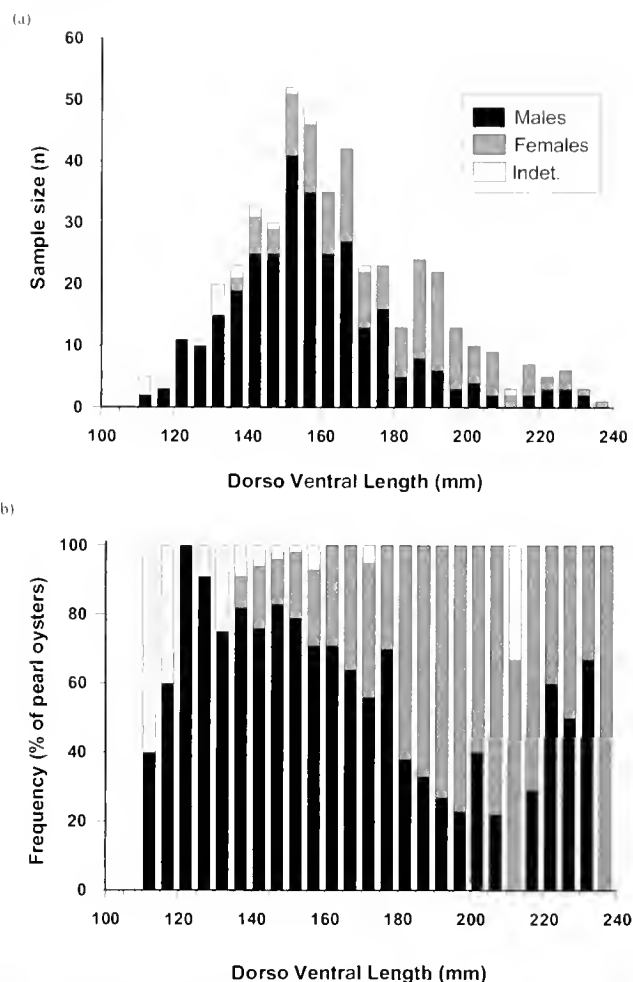


Figure 10. Sex-ratio by size in wild stocks of *Pinctada maxima* from 80 Mile Beach (Zone 2). (a) sample size per size-increment; (b) % frequency by size-increment.

location specific, and generally low, with indirect estimates from length-converted catch-curve analysis (0.11–0.18) being higher than direct estimates from tag-recapture studies (0.02). Sims (1992) estimated natural mortality at 0.11 for *Pinctada margaritifera*, which suggests that low rates of  $M$  for pearl oysters are to be expected. The differences between  $M$  was primarily related to depth, and shallow areas of the pearl stocks may exhibit a higher rate of natural mortality caused by increased turbidity and greater water movement and susceptibility to annual extreme storm events, such as cyclones and perhaps higher predation. We hypothesize that *P. maxima* in Western Australia, a sedentary species having evolved under the influence of huge tides and cyclones would be favored by having low natural mortality as a life-history strategy, indicating a robustness to cope with extreme environmental conditions.

Historically, varying catch per unit effort (CPUE in shells caught per diver hour) in the pearl oyster fishery (Fig. 11), suggests that recruitment is quite variable, a conclusion in common with many marine invertebrate species. Whereas the introduction of GPS (Global Positioning System) in 1994 might have confounded the changes in CPUE occurring at that time, subsequent changes in CPUE suggest that fishery performance can reflect recruitment variation. Understanding this natural variation in recruitment will greatly enhance our management capability of this fishery, and our preliminary work on using the "piggyback" spat recruitment index has yielded promising results. Firstly, it was possible to collect adequate samples of newly settled spat as part of the on-board vessel monitoring program and

divide them up into two age classes (0+ and 1+). The results proved consistent from year to year because the fishery largely operates within the same 3 mo each year (mid-March to mid-June). Secondly, preliminary predictions detected a future temporal vari-

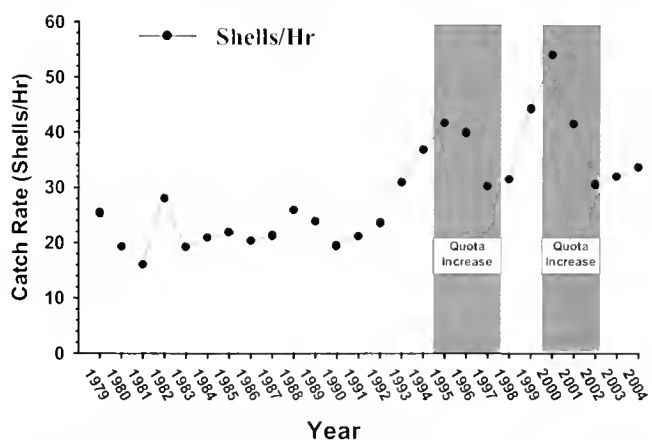


Figure 11. Catch rate (shells caught per diver hour) in the Western Australian *Pinctada maxima* fishery (Zones 2 and 3 combined) between 1979 and 2004.

ability in CPUE, which is consistent with the past history of CPUE in the fishery, namely that the general pattern has been 2–4 y of “baseline” CPUE around 30 shells per hour, followed by 2–3 y of increased CPUE, up to 60% above the baseline and then a drop back to normal levels. Future work will quantify effects of habitat and depth and other environmental factors, such as variables representing larger oceanographic conditions (e.g., ENSO phenomena) and provide a formal predictive index to assist in managing level of catch.

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## IN PURSUIT OF COST-EFFECTIVE FISHERIES ENHANCEMENT OF NEW SOUTH WALES BLACKLIP ABALONE, *HALIOTIS RUBRA* (LEACH) FISHERY

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**ABSTRACT** The diverse and often complex essential components of successful marine stock enhancement are briefly reviewed. Progress on a project to enhance the New South Wales blacklip abalone (*Haliotis rubra*) fishery is discussed to demonstrate that for sedentary reef invertebrates such as abalone, successful enhancement entails an understanding of recruitment and production limiting factors. These include age and size specific growth and mortality rates. These variables encompass the influence of other species within reef ecosystems, especially predators and species that compete directly with them for space, shelter and food. This discussion is also used to demonstrate that such difficult projects can be facilitated and refined using a simple bio-economic model centered on two interdependent tasks. The first is to minimize net costs per unit of additional sustainable production generated by enhancement. The second is to determine the minimum amount of additional sustainable production required to render a project cost-effective. This first task has, as expected, proven complex and its resolution protracted and expensive, requiring innovative biotechnology and a comprehensive knowledge of the natural biology of blacklip abalone. This discussion principally reflects the perspective of the direct potential beneficiaries of abalone fisheries enhancement, namely commercial and recreational fishers and does not deal extensively with wider views and interests of other stakeholders, namely relevant government agencies and NGOs and the general public.

**KEY WORDS:** fisheries enhancement, abalone, *Haliotis rubra*

### INTRODUCTION

#### *Generalized Theory and Practice of Marine Fisheries Enhancement*

Blankenship and Leber (1997) identified 10 key components as essential for responsible marine stock enhancement. These included the need to: (1) prioritize and select target species for enhancement; (2) develop a species management plan that identifies harvest opportunity, stock rebuilding goals and genetic objectives; (3) define quantitative measures of success; (4) use genetic resource management to avoid deleterious genetic effects; (5) use disease and health management; (6) consider ecological, biological and life-history patterns when forming enhancement objectives and tactics; (7) identify released hatchery seed and assess stocking impacts; (8) use an empirical process for defining optimum release strategies; (9) identify economic and policy guidelines and (10) use adaptive management. Blankenship and Leber (1997) reviewed three finfish case studies to verify that this responsible approach to marine stock management is practical.

In a recent review of the 100 y history of fisheries stock enhancement projects, Molony et al. (2003) concluded that most have had little or no demonstrated success. They ascribe this to undue preoccupation with seed production technology and failure to identify and/or control underlying reasons why the targeted fisheries were underperforming or not meeting management objectives. They also concluded that stock enhancement has often been applied in isolation from other fisheries management tools and have ignored broader ecosystem perspectives. To address these shortcomings, the authors proposed a flow-chart implementation model comprising four sequential steps: (1) review all relevant ecological and stock status management fisheries information; (2) make a comparative evaluation of all relevant fisheries management tools with a potential to meet targeted objectives; (3)

instigate a scientifically based pilot enhancement program with clear appropriately targeted objectives and (4) if warranted by outcomes of the pilot program, initiate a follow-up full scale commercial program.

What follows is a discussion of a research project that has attempted to achieve sustainable and cost-effective enhancement the blacklip abalone (*Haliotis rubra*, Leach) fishery in New South Wales (NSW), using hatchery-produced seed. This research, much of which is reported in more detail by Heasman et al. (2004) in a contract research report, has collectively addressed most of the important issues and elements identified by Blankenship and Leber (1997). Its implementation over a 3-y period generally conformed to the first three sequential steps prescribed by Molony et al. (2003). Towards the end of the third year of pilot seeding operations (step 3), an extensive reappraisal of the project was made. This comprised a repeat of steps 1 and 2 (review of all other relevant fisheries biology and management information) and incorporated a wealth of practical experience gained, together with findings of an extensive array of complementary laboratory and field-based larval and juvenile seeding experiments. It also culminated in the development of bioeconomic benefit and cost and risk assessment model.

#### *Previous Attempts to Enhance Wild Stocks of Abalone With Hatchery-Produced Seed*

The efficacy of enhancing wild stocks of abalone with hatchery-produced seed has been investigated internationally and within Australia. As stated by Shepherd et al. (2000), "Release of larvae and juveniles has often been proposed as a panacea for rehabilitating depleted or over exploited reef." Seeding of juveniles has been practiced in Japan for several decades with some success (Kojima 1995, Masuda & Tsukamoto 1998). Larval release has been practiced sporadically in Mexico since the 1960s (Ortiz-Quintanilla 1980). Prior to this study, research in NSW into the efficacy of previously developed techniques for the release of seed abalone was limited to larval releases in Twofold Bay by a company Abalone Shellfish Enterprises Pty Ltd. (Keesing et al. 1994).

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Note: these budgets incorporate new highly efficient hatchery and nursery technologies developed in this study but do not include depreciation on plant and equipment.

Although survival of the larvae was not monitored, a localized high density aggregation of sublegal size abalone was observed at one release site several years later (Ross Werner, pers. comm. 1998). This information, though anecdotal, encouraged collaborative involvement of total allowable commercial catch (TACC) quota holders in this project as mediated through the NSW abalone management and advisory committee (ABMAC).

Larvae of *H. rubra* and greenlip abalone (*Haliotis laevigata*, Donovan) were released at different densities in South Australia by Preece et al. (1997). Generally low and strongly density-dependent survival in the range 0.02% to 7.8%, occurred 6–7 d after settlement. Such strongly density-dependent rates of mortality of larvae and early postlarvae were also reported by McShane (1991) in relation to naturally recruited stocks of *H. rubra* in north eastern Victoria. On the basis of these reports, Shepherd et al. (2000) recommended against larval seeding, as did Schiel (1993), based on similar results with seeding of *H. iris* (Paua) larvae in New Zealand.

In a review of experimental releases of 7-mo-old (12 mm) hatchery-produced *H. rubra* in Tasmania, Shepherd et al. (2000) concluded that very high rates of mortality over the first few weeks were due mainly to handling stress. They also concluded that high mortality up to 1 y after release was caused by persistent density-dependent mortality factors and predator naivety.

#### Status of the NSW Abalone Fishery and Scope for Enhancement

The NSW abalone fishery is based entirely on *H. rubra* and is largely confined to the southern half of the state. Annual catch (Fig. 1) peaked at about 1200 t in 1971/1972, and remained above 600 t through to the early 1980s. Since 1973 a succession of fishery management initiatives (Fig. 1) have been implemented. These have included a reduction in effort through license regulation, limiting catch by quota allocation and introduction of size limitations. Regional fishing closures were also imposed between Port Stephens (32°42'S; 152°10'E) and Jervis Bay (35°03'S; 150°44'E) after a major depletion of stocks by the disease *Perkinsus* between 2000 and 2002. A TACC of 370 t was first introduced in 1989. Subsequently it has been progressively reduced to 333 t in 1996, to 305 t in 2000 and down to its current level of 281 t in 2003 (NSW Fisheries 2004). Subsequently, it was further reduced to 208 t in 2004 and to 130 t in 2005.

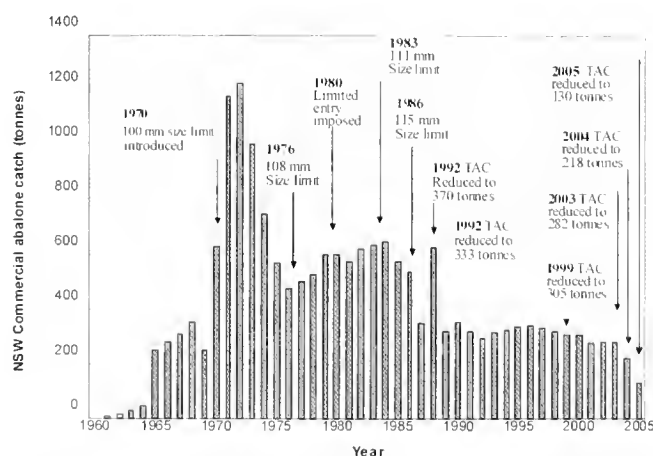


Figure 1. Annual commercial catch data and management initiatives for the NSW *H. rubra* fishery since its inception in 1960. (Source data: Anon 2002; NSW Fisheries 2004.).

A common anecdotal report of *H. rubra* fishers over this 30-y period is that many formerly productive areas of reef, typified by foliose algae (seaweed) and associated complex communities of fish and invertebrates including abalone, have undergone a transition to "barrens." These bare rock areas typically carry high densities of the black (=purple) sea urchin, *Centrostephanus rodgersii*. (J. Smythe, pers. comm.). Such observations are compatible with the findings of Andrew and Underwood (1992) that densities of *H. rubra* and *C. rodgersii* are negatively associated and by the finding of Shepherd (1973) that continuous grazing pressure, exerted by dense aggregations of *C. rodgersii*, can degrade complex community reefs. Andrew and O'Neill (2000) and Worthington and Blount (2003) estimate that barrens habitat constitute an average of 40% to 50% of near-shore reef areas in NSW.

Whereas the massive extent of barrens habitat in NSW appears to offer scope for rehabilitation of depleted *H. rubra* stocks, natural recolonization is probably limited by the combination of competitive exclusion by *C. rodgersii* and the very restricted dispersal of *H. rubra* larvae from their parents (Prince et al. 1988). Hamer (1982) and Andrew et al. (1998) demonstrated that habitat improvement by way of culling urchins from urchin dominated reef in Southern NSW resulted in rapid recovery to abalone densities typical of high producing reef within 3–4 y. The recovery process also included a dramatic increase in the coverage and biomass of foliose algae and an associated increase in biodiversity of floral and faunal assemblages. Nevertheless, urchin culling is very labor-intensive and colonization is limited to small localized areas. Accordingly, the task of culling *C. rodgersii* from up to 50% of coastal reefs commercially fished for abalone in NSW, that collectively comprise about 5000 ha, is daunting.

By contrast, mass hatchery production and release of seed *H. rubra* provides a potentially powerful means of rapidly enhancing depleted abalone subpopulations over extensive areas. Seeding also addresses other factors limiting recruitment and consequentially sustainable yields. Such factors include the combined effects of commercial, recreational and illegal fishing pressure and diseases such as *Perkinsus* that has devastated stocks north from Jervis Bay to Port Stephens (Worthington 2002). Other recruitment limiting factors are pollution and competitive exclusion especially of settlement stage larvae and small postlarvae by a diverse array of large common surface grazers. These comprise other gastropods including several ubiquitous turban shell species and a comparable array of urchins additional to *C. rodgersii*. In a recent experiment (Heasman unpublished data) high densities of the common tent shell (*Astraliun tentoriformis*) or of *Turbo torquatus* in high densities were found to reduce yields of 1-wk-old *H. rubra* postlarvae, seeded as larvae onto natural CCA (crustose coralline algae) rock settlement substrates, by 98% and 94% respectively. In the same experiment, the presence of either black urchins (*C. rodgersii*) or adult abalone reduced postlarval yields by similarly high margins of 90% and 78% respectively.

#### SEED PRODUCTION AND DEPLOYMENT TECHNOLOGY

##### Improved Year-round Availability of Ripe Broodstock for Induced Spawning and Hatchery Production

Attempts to immediately produce viable eggs from wild-caught adults were unsuccessful, with a significant number of viable eggs (1.5 million) being produced on only 1 of 42 occasions. Over the same period seven successful inductions of spawning were

achieved using broodstock acclimatized over longer periods in ambient flow-through seawater tanks. These yielded 26.8 million eggs (mean fecundity 1.12 million eggs/spawner) that in turn yielded 13.3 million competent larvae. However, these spawnings and subsequent hatchery operations occurred at irregular intervals and were largely limited to the spring to early summer natural breeding season of *H. rubra* in NSW.

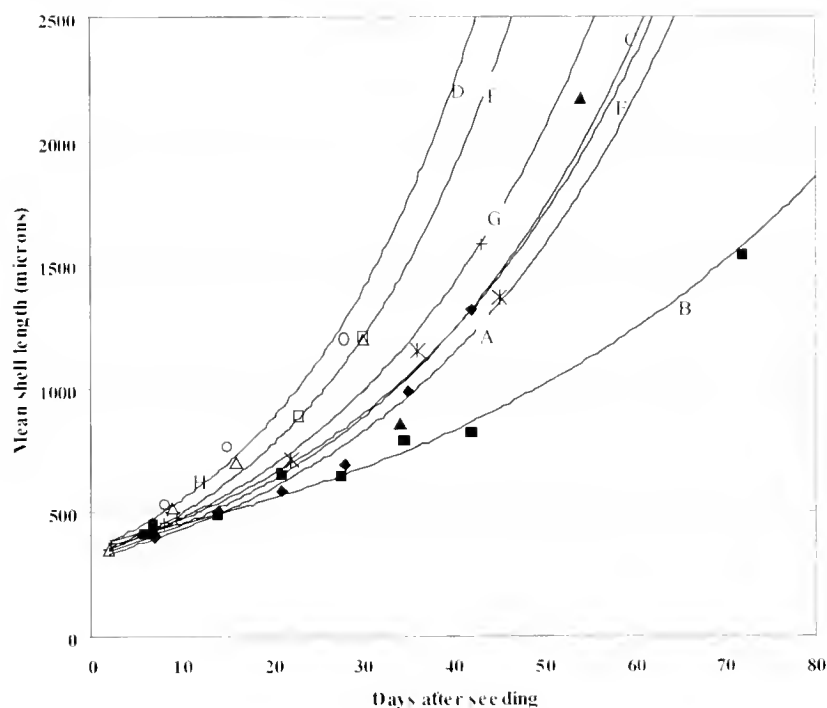
For the first time in Australia, greatly improved access to ripe, ready-to-spawn broodstock was achieved with captive stock conditioned in a recirculating seawater system operated at  $15 \pm 2$  °C. These stock were originally collected from seven localities between Port Stephens, 150 km north of Sydney, to Disaster Bay ( $37^{\circ}15'$ ;  $149^{\circ}58'E$ ) near the Victorian border. Half (17) of 34 spawning induction operations, spread throughout the year, were successful and yielded 59.3 million eggs. Mean spawning response rate using these conditioned broodstock was 11% (85 out of 785 females). Mean fecundity ( $\pm$  s.e.) was  $1.25 \pm 0.49$  million eggs/spawner (range of 42,000–4.0 million). A total of 24.0 million competent larvae were produced and used for larval seeding experiments or for nursery production of juvenile seed and related experiments. Yields of 7–8-d-old competent larvae from eggs averaged 40% (range 8% to 71%), which was consistent with rates

routinely reported by commercial hatcheries in Australia. Subsequent yields of 6–9 mo old juvenile seed from competent larvae averaged 5.7% and varied greatly from 8% to 75%.

#### Hatchery and Nursery Technology Development

The low and inconsistent yields of juveniles raised the need for better knowledge of and control over nursery production. A mean shell length of about 1.5 mm was identified as the minimum size needed to ensure trouble free weaning of postlarvae onto finely ground particulate diets (Adam and Amos P/L, South Australia). Postlarvae grew at an exponential rate regardless of season or seed density (Fig. 2). Plate residence time from settlement to 1,500  $\mu$ m ranged widely. Batches reared in summer/early autumn required only 30–35 d. At the other extreme, winter and early spring reared batches required 50–72 d. Growth rates thus appeared primarily dependent on seasonal temperature.

An important feature of growth on diatom plates is that it continues exponentially until either food runs out (plates grazed out), in which case it abruptly stops, or when postlarvae reach a mean shell length of 1–2 mm at which time they can be successfully transferred to raceways for weaning onto artificial diets. As



Batch	Larval seeding density	Spawning Date	Growth season	Period to min. mean weaning size of 1500 microns	Mean growth rate microns/day	Best fit regression	R <sup>2</sup>
A	5882 plate	23/07/98	late Winter early Spring	50 days	23 $\mu$ m/day	$y = 314.71e^{0.0322x}$	0.9813
B	5147 plate	27/08/98	early to mid Spring	72 days	17 $\mu$ m/day	$y = 373.24e^{0.02x}$	0.9751
C	1838 plate	25/12/98	Summer	45 days	26 $\mu$ m/day	$y = 323.15e^{0.0335x}$	0.9808
D	7625 plate	20/01/99	late Summer	32 days	36 $\mu$ m/day	$y = 348.05e^{0.0464x}$	0.9731
E	1472 plate	6/09/99	early to mid Spring	52 days	22 $\mu$ m/day	$y = 345.04e^{0.0319x}$	0.9921
I	735 plate	14/09/99	mid to late Spring	47 days	24 $\mu$ m/day	$y = 320.56e^{0.0442x}$	0.9999
G	1323 plate	24/11/99	early to mid Summer	41 days	28 $\mu$ m/day	$y = 333.86e^{0.0362x}$	0.9988
H	2507 plate	1/02/00	late Summer	35 days	33 $\mu$ m/day	$y = 337.31e^{0.0431x}$	0.9923

Figure 2. Variation in growth of eight commercial scale batches of *H. rubra* postlarvae grown on conventional diatom plates. Note: growth was exponential in all cases (Heasman et al. 2004).

illustrated in Figure 3, production batches that were inadvertently allowed to exhaust diatom films after having reached the minimum weaning size, stopped growing and starved before being harvested from nursery plates. Substantial and sometimes catastrophic losses of postlarvae occurred as a consequence of such episodes. This was probably caused by combined stresses of starvation, anaesthesia and handling when harvested and transferred to shallow exposed raceway habitats for weaning. The size and age at which growth stopped and starvation began appeared inversely related to initial density of competent larvae seeded onto the plates.

#### Results of Experiments Conducted to Improve the Reliability and Cost Efficiency of Large-Scale Production of Juvenile *H. rubra* Seed

Results of one experiment to investigate effects of variable larval density of 500–4,000 larvae per plate ( $0.15\text{--}1.2\text{ cm}^{-2}$ ) on settlement, metamorphosis and subsequent growth on conventional nursery plates showed that yields of postlarvae (Fig. 4) peaked after 4–7 d. They also showed that yields increased from 26% to 71% with increasing seeding density. However, from day 7–28 after seeding, survival across all seeding densities progressively converged to a common rate of about 20% that persisted until the termination of the experiment at day 56. Growth (Fig. 5) remained independent of initial seeding density up to 14 d, averaging about  $14\text{ }\mu\text{m}$  per day. Subsequent growth became progressively more density dependent. By day 56, growth rates at residual densities of 10, 100 and 1,000 postlarvae per plate averaged about 40, 30 and  $22\text{ }\mu\text{m/day}$  respectively. Minimum mean shell lengths of about  $1,400\text{ }\mu\text{m}$  at day 56, attained at the highest initial density of 4,000 postlarvae per plate, were nevertheless found to be acceptable for the purpose of early weaning of postlarvae. These improved techniques provide scope to increase output per plate from one to at least four batches per year and to increase yield densities to a consistent range of up to 500–1,000/plate, thereby greatly raising annual yield/plate/year up from 30–80 up to a range of 2,000–4,000.

In another experiment, effects of temperature on settlement, metamorphosis and early postsettlement growth and survival were investigated. Settlement rates on natural CCA coated rock settlement substrates ranged from 20% to 40%. Peak settlement and

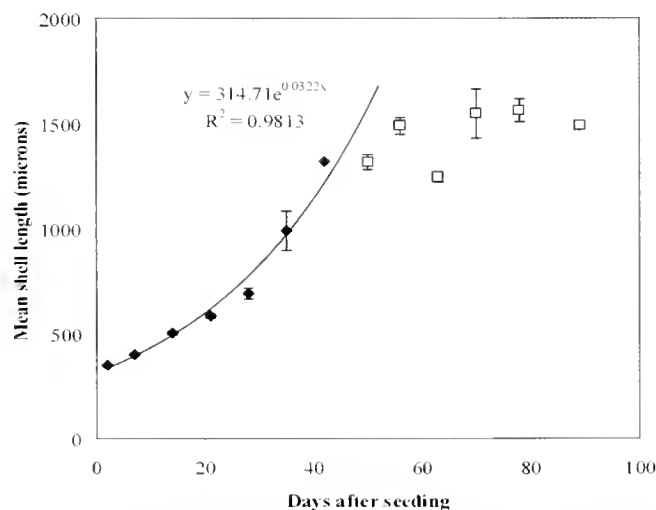


Figure 3. Effect of over-grazing on growth of *H. rubra* postlarvae seeded onto conventional diatom plates (Heasman et al. 2004). Note: initial exponential growth (solid circles) succeeded by stalled growth and starvation (open circles). Bars are  $\pm$ SE of means,  $n = 30$ .

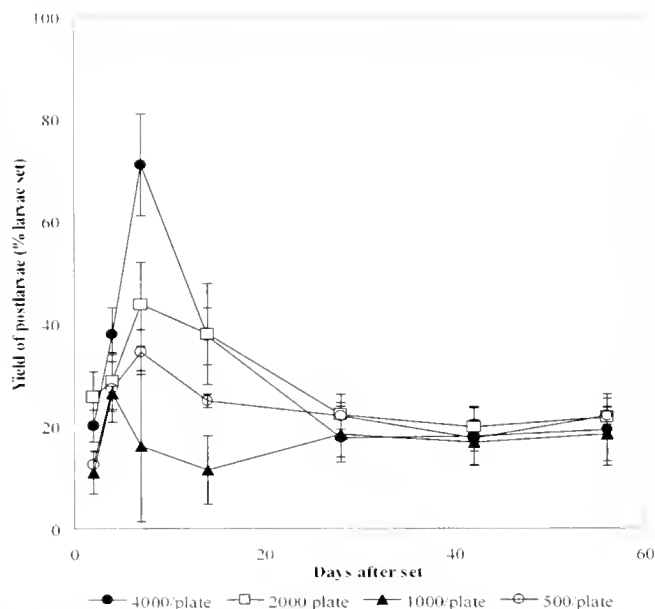


Figure 4. Effect of larval seeding density on the relative yield of *H. rubra* postlarvae seeded onto diatom plates. Bars are  $\pm$ SE of means,  $n = 4$  (Heasman et al. 2004).

yields of postlarvae occurred at about  $17^\circ\text{C}$  (Fig. 6). During the first week after settlement growth rates of postlarvae (Fig. 7) increased progressively from zero at  $10^\circ\text{C}$  to a peak of  $\sim 30\text{ }\mu\text{m/day}$  at  $23^\circ\text{C}$ , but then fell dramatically back to zero with a further rise in temperature to  $27^\circ\text{C}$ . Effects of temperature on growth of four juvenile age/size classes of *H. rubra* (Fig. 8 a to d) showed that further downward shift in optimum temperature from about  $17^\circ\text{C}$  to  $15^\circ\text{C}$  occurs with progressive age and size in juvenile *H. rubra*. Results also demonstrated that moderate to high rates of settlement and subsequent growth and survival could be achieved year-round on the central coast of NSW at mean monthly sea temperatures in the range  $16^\circ\text{C}$  to  $22^\circ\text{C}$  but that successful year-round hatchery-ambient temperature nursery production would be limited at more southern sites subject to sea temperatures below about  $14^\circ\text{C}$ .

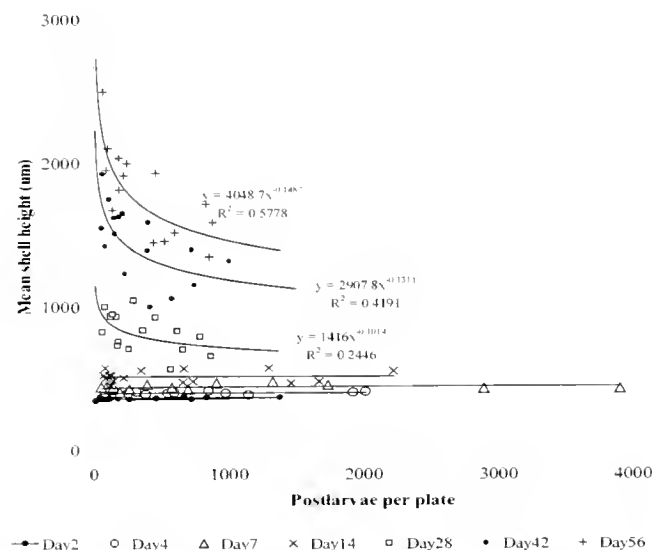


Figure 5. Effect of density on growth of *H. rubra* postlarvae on diatom plates 2–56 days after seeding as larvae (Heasman et al. 2004).

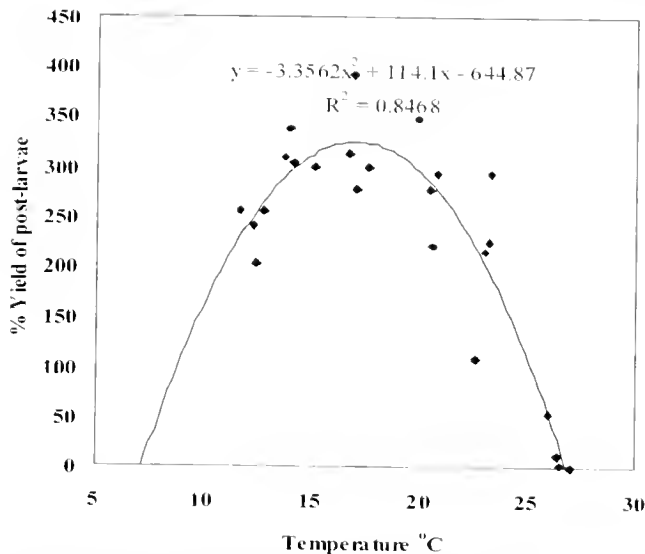


Figure 6. Effect of temperature on yield of day 6 *H. rubra* postlarvae as a percentage of larvae seeded onto crustose coralline algae coated rocks (Heasman et al. 2004).

#### Transportation and Storage of Juvenile *H. rubra*

The need to transport and deploy batches of up to several hundred thousand 6–9-mo-old (5–15 mm) juvenile *H. rubra* up to 500 km from the hatchery prompted the development of suitable storage and transportation methods. Key issues addressed were whether stock should be stored and transported wet or damp, optimum temperatures for storage and transportation and the maximum duration over which they can be safely stored and/or transported without compromising the postrelease vigor and survival. Juvenile *H. rubra* (mean SL 14 mm) previously held at ambient temperatures of 18°C to 22°C survived at acceptable rates (i.e., above 80%) for up to 48 h when stored damp in pure oxygen between 13°C and 16°C. The best 48 h survival rate of 95% was achieved with damp storage at 14°C. Generally poorer rates achieved with wet transportation were ascribed to declining physiochemical conditions, especially dissolved oxygen that rapidly fell below an acceptable threshold of 95% saturation.

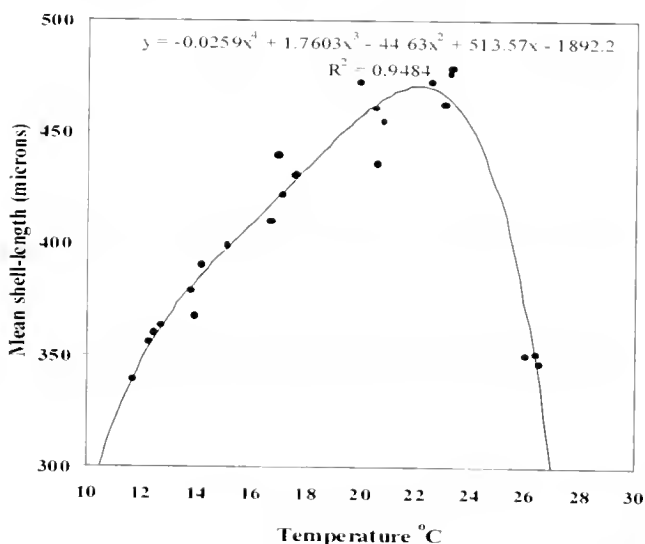


Figure 7. Effect of temperature on growth of day 6 *H. rubra* postlarvae seeded onto crustose coralline coated rocks (Heasman et al. 2004).

#### Field Deployment and Postrelease Growth and Survival

Improved methods of seeding hatchery-produced *H. rubra* larvae and juveniles onto natural reefs were developed, and the effectiveness of these methods to deliver competent larvae and juveniles to reefs tested. Several alternative methods of monitoring the settlement and early survival of larvae, postlarvae and juveniles within their respective release areas were developed. Field experiments were used to demonstrate the ability of juveniles to rapidly disperse from points of release and provided greater insight into factors that must be considered when attempting to estimate long-term survival of seeded stock.

In excess of 24 million larvae and nearly 900,000 juvenile *H. rubra*, most falling within the "button" mean size range of 6–18 mm SL, were seeded to 57 sites within six locations along the NSW coast. The survival and growth of larvae and juveniles were monitored for periods up to 30 mo. Short-term (<2 mo) sampling was used to investigate movement of juveniles and to compare alternative sampling techniques. The best survival for seeded larvae a year or more after release was 125 out of 1.1 million (1 in 8,800) recorded 553 days after release. As in earlier abalone seeding studies (Preece et al. 1997) seeding of ready-to-settle larvae was shown here to be fraught with practical difficulties and with very low rates of survival projected to yield less than 1 in 20,000 to a legal shell length of 115 mm.

Juveniles were mainly deployed as clusters of 700–2,500 in predator protective release devices initially comprising CCA coated rocks within wire mesh cylinders. These were superseded by rectangular boxes comprising sections of PVC down-pipe. Mean survival rates 1–2 y after release ranged from 0% to 4%. Average growth rates were similar among and between sites with juveniles expected to reach maturity (>90 mm SL) after 2.5–3.5 y, and projected to reach legal SL of 115 mm after 4 y. These low survival rates prompted a compilation and analysis of published age and size-specific mortality data for *H. rubra* (Table 1, Fig. 9) that were used to estimate yield per recruit and related parameters for a wide range of age/size classes of seed from competent larvae to 6-y-old adults (Table 2). A review of published ecological information on *H. rubra* revealed that, although 1–4-wk-old wild postlarvae occur at natural densities of up to several 1000/m<sup>2</sup>, and 6-mo-old juveniles at up to 30/m<sup>2</sup>, survivors of these groups converged to a common narrow density range of 1–3/m<sup>2</sup> as 15–30 mm, 1+ year olds (Prince et al. 1988, McShane 1991, Shepherd et al. 2000). It was then postulated that if natural survival rates of 6–9-mo-old button size (7–15 mm) juveniles, through to the minimum legal size, of 1 in 20–30 are to be achieved with hatchery-produced seed, then such seed should be sparsely deployed at densities matched to the food based carrying capacity of CCA rock habitats of this size/age class of juveniles, rather than in large dense clusters as previously practiced.

#### Evaluation of Postlarvae as an Alternative to Competent Larvae for Seeding

Major practical advantages of using week old postlarvae rather than settlement stage larvae for seeding operations were also identified in three laboratory experiments. Results (Fig. 10) showed that postlarvae can be readily seeded at very high densities onto small CCA coated pebbles (vector rocks) and retained for up to 8 d without significant restriction of growth or ability to rapidly disperse into simulated small boulder habitats after release. Carrying capacity of CCA rock habitat declines reciprocally with exponential increases in the biomass of individual *H. rubra*, falling to

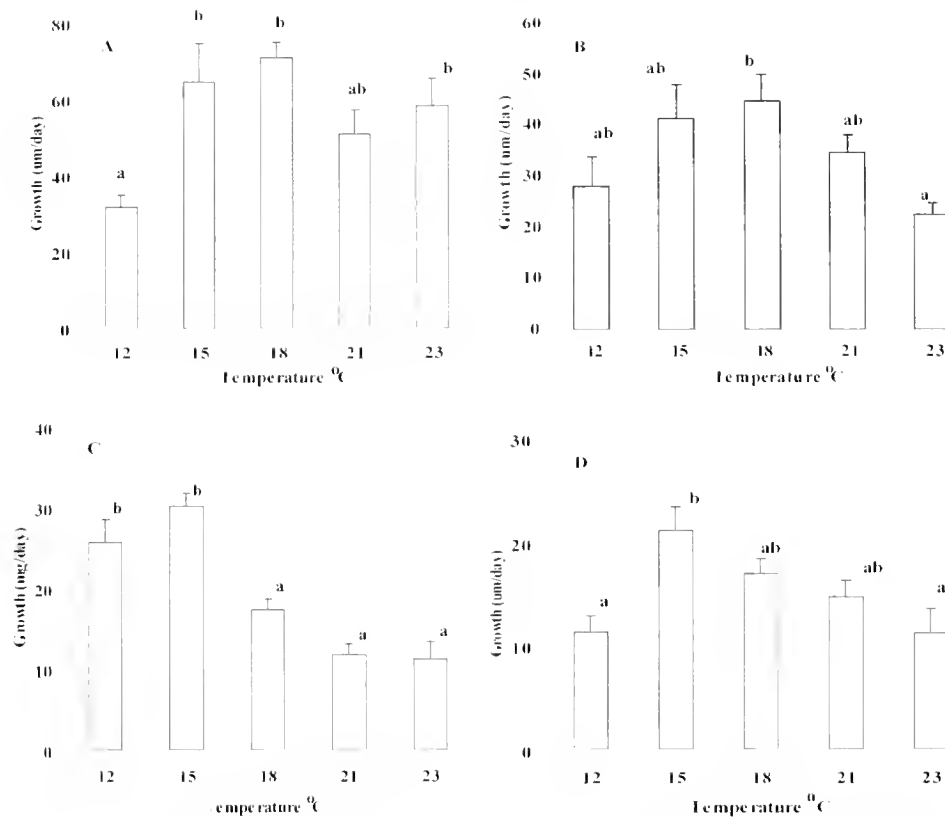


Figure 8. Effect of temperature on growth (a) in shell length of 3 mo old, 2–6 mm; (b) in shell length of 7 mo old, 6–8 mm; (c) in biomass of 17 mo old, 20–25 mm and (d) in shell length of 30 mo old, 50–60 mm, juvenile *H. rubra*. Bars are  $\pm$ SE,  $n = 4$  (Heasman et al. 2004).

densities levels of 1,000–2,000/m<sup>2</sup> for 2-mo-old (1.5 mm) postlarvae (Fig. 11), and by extrapolation, to levels of around  $\leq 30$ /m<sup>2</sup> for 6-mo-old (7 mm) juveniles. The latter was consistent with above cited peak densities reported for equivalent wild *H. rubra*. Surface grazing on CCA thallus and associated epibiota remains the primary food source of juveniles up to at least 10 mm SL and is only then progressively supplanted by drift seaweed over the following

1–2 y in individuals up to at least 35 mm SL (Shepherd & Daume 1996). An important aspect of these findings is that they were consistent with the earlier-cited convergence of 1+-y-old wild juveniles to densities of 1–3/m<sup>2</sup>. These observed carrying capacity limitations of CCA rock habitats occurred in the absence of other important density and survival limiting factors, namely competition from other surface grazers such as urchins and other gastro-

TABLE 1.

Published age related mortality data for wild *Haliotis rubra* (See Shepherd & Breen 1992, for the mathematical relationship between instantaneous and proportional mortality).

Mean Age (Age Span)	Survival for Period or Per Year	M (Annualized Instantaneous Natural Rate of Mortality)	Source
25 days (0–49 days)	0.5%	311/y	Preece et al. 1997
80 days (1 week to 5 months)	3%	6.6–10.2 (8.4)/y	McShane 1991
4 months (1 week to 8 months)	5.5%	1.4/y	Prince et al. 1988
1.25 years (6 months–2 years)	20%	0.7/y	Prince et al. 1988
2 years (1.5–2.5 years)	44% per year	0.81/y	Day & Leorke 1986
2.25 years (2 to 2.5 years)	41% per year	0.9/y	Prince et al. 1988
2.5 years (2 to 3 years)	64% per year	0.45/y	Hamer 1982 (Botany Bay stock)
3 years (2–4 years)	66% per year	0.42/y	Shepherd & Breen 1992
3.3 years (3 to 3.5 years)	45% per year	0.8/y	Prince et al. 1988
3.5 years (2–5 years)	70% per year	0.36/y	Shepherd et al. 1982
4 years (2–6 years)	81% per year	0.21/y	Shepherd et al. 1982
4.3 years (4 to 4.5 years)	74% per year	0.3/y	Prince et al. 1988
4.5 years (4 to 5 years)	76% per year	0.274/y	Hamer 1982 (Eden Stock)
5+ years	82% per year	0.20/y	Bemssen & Powell 1979
5.3 years (5 to 5.5 years)	90% per year	0.1/y	Prince et al. 1988
6.3 years (6 to 6.5 years)	78% per year	0.25/y	Prince et al. 1988



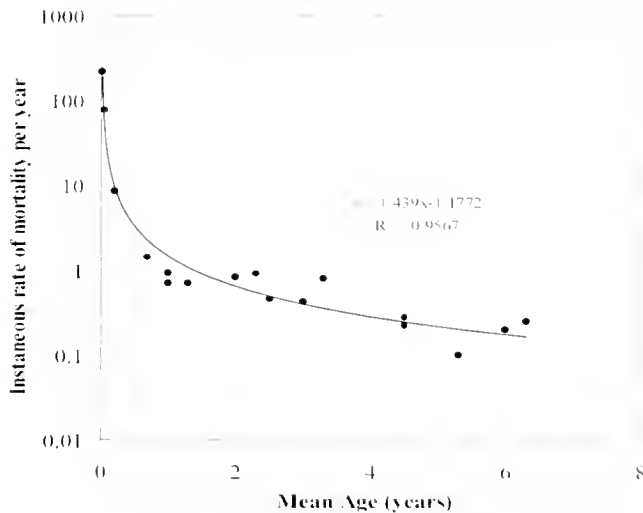


Figure 9. Age related annualized instantaneous mortality rates for wild *H. rubra* (see Table 1 for source of data).

pods and predation especially by finfish especially wrasses (Shepherd & Turner 1985, Shepherd 1998), wirra cod (*Acanthistius ocellatus*) and morwong (*Cheilodactylus fuscus*) (Heasman, unpublished observations) and large invertebrates including starfish, rock lobsters, large crabs and octopus (Shepherd & Breen 1992, Shepherd 1998). Accordingly, the carrying capacity of CCA rock habitats can be largely ascribed to nutritional factors in juveniles up to 10-mm SL and an important but diminishing factor in larger juveniles to 35-mm SL and around 18 mo of age.

On the strength of these findings, a final reef seeding experiment was conducted at Disaster Bay near the NSW/Victorian border. Two deployment treatments; the first consisting of 10 evenly spread clusters of 100 juveniles, averaging 15-mm SL, and the second a central cluster of 1,000 juveniles of the same size, were each deployed over three replicate 113 m<sup>2</sup> sites. This represented a targeted seeding density of less than 10 juveniles/m<sup>2</sup>, typical for this size/age class of juveniles on healthy productive reef (Prince et al. 1988, McShane 1991, Shepherd et al. 2000). A year later, mean survival across sites seeded with 10 × 100 juveniles was within the expected range for equivalent wild stock, namely 12.5% (range 5% to 23%), whereas that of the alternative clustered treatment was only 3.8% (range 0% to 9%).

#### DEVELOPMENT OF A BIO-ECONOMIC MODEL FOR ENHANCING THE NSW ABALONE FISHERY

Many of the earlier mentioned findings were used to develop a comparative bio-economic model for *H. rubra* fisheries enhancement using alternative age/size classes of seed. The model is based on the most fundamental of economic equations:

$$\text{Profit} = \text{Revenue} - \text{Total costs}$$

where:

Revenue = long-term average beach price for abalone  
× additional sustainable catch generated by seeding

Total costs = costs of all elements of production and deployment of seed but not including those of monitoring the wider environmental consequences of seeding, especially impacts on plant and animal communities and on the genetics of extant wild *H. rubra* populations.

Key inputs, assumptions and rationale thereof associated with this model as follows:

- Additional sustainable annual catch targeted is 300 t (≡ 1 million, 120 mm abalone @ 300 g). This is valued at AU\$15M based on an assumed average beach price of AU\$45/kg (AU\$15 ea.) (ABARE 2003). The rationale for nominating an enhancement target of an additional 300 t/year, is that it would restore the total commercial catch to a range of 500–600 t/year, which approximates the median annual catch of the fishery since its inception in 1960 (Fig. 1). It is likely that enhanced production of *H. rubra* can only occur at the expense of other reef surface and drift seaweed grazers that compete with *H. rubra* for food and space. However, when it is considered that 300 t of additional *H. rubra* equates to about 0.6% of the biomass of its most important competitor, the black sea urchin, *C. rodgersii*, estimated at 40,000–50,000 t by Worthington and Blount (2003), such impacts are likely to be sustainable.
- The assumed mean age and weight of released abalone at harvest are 5 y and 300 g respectively. This estimate constitutes a median growth rate for *H. rubra* populations throughout NSW, based on regional specific rates originally derived from tag and recapture size data reported by Hamer (1982) and re-evaluated by Worthington and Andrew (1997).
- The five size/age classes of seed (Tables 2–3) and associated assumptions were as follows:
  1. *Competent larvae*. It was assumed that only 1 in about 20,000 seeded larvae would survive to market size adults in compliance with yield per recruit predictions presented in Table 2. The latter, including a mortality rate of ~95% in the first week, are based on published instantaneous mortality data for wild *H. rubra* presented in Figure 9 and assume that seeding does not significantly reduce natural recruitment. Apart from enormous logistical problems of deploying the requisite 20 billion larvae over hundreds of hectares of juvenile habitat by SCUBA under calm sea conditions, the production of the 2 billion larvae required would entail the reproductive conditioning and induced spawning of about 30,000 captive female broodstock (Table 3). The latter is considered impractical if not cost prohibitive.
  2. *0.4-mm postlarvae*. It was assumed that postlarvae would be preseeded onto vector rocks thereby circumventing otherwise prohibitively costly and difficult production and deployment problems plus very high postrelease mortality losses sustained by larvae and postlarvae during the first week in the wild. An assumed yield per recruit rate of 1 harvestable *H. rubra* per 2,000 seeded postlarvae is based on data presented in Table 2 and incorporates an experimentally determined postsettlement mortality rate of 50% in the absence of predators. This particular mode of seeding would require year-round reproductive conditioning and induced spawning of about 3,000 captive female broodstock (Table 3). The latter, although daunting, is considered practicable and affordable. The most critical limitation of this seeding strategy is uncertainty surrounding the fact that it has not been experimentally trialed in the field let alone on a commercially significant scale.
  3. *2-mm postlarvae*. An estimated yield per recruit of 1 harvestable *H. rubra* per 157 ex-diatom nursery plate 2 mm postlarvae is based on reported age specific natural mortality data presented in Table 2. Production of requisite 157 mil-

TABLE 2.  
Estimated age related survival, yield per recruit and associated parameters for wild *H. rubra*.

Age (post set and size (mm))	Assumed Rate of Instantaneous Natural Mortality (M)	Proportion Surviving Period	Cumulative Proportion Surviving from Larvae	Number of Larvae Required to Yield 1 Abalone to This Age	Proportion from This Age Living to Legal Size	Number of Seed of This Age Needed to Yield 1 Legal Size Abalone	Number per Million Seed Deployed at This Age That Will Reach Harvest Size	Source of Survival Data
Spawning to competent larvae	?	?						
Competent larvae to 8 days post set (0.3–0.4 mm)	0.44/day	0.03	0.0300	33	0.0000	20704	48	Preece et al. 1997
8–19 days post set (0.4–0.6 mm)	0.042/day	0.63	0.0189	53	0.0016	621	1610	Preece et al. 1997
0.5–1.5 months (0.6–2.0 mm)	0.9/month	0.4	0.00756	132	0.0026	391	2556	McShane 1991
1.5–2.5 months (2–4 mm)	0.7/month	0.5	0.00378	265	0.0064	157	6389	McShane 1991
2.5–3.5 months (4–6 mm)	0.6/month	0.55	0.00208	481	0.0128	78	12778	McShane 1991
3.5–4.5 months (6–8 mm)	0.5/month	0.61	0.00127	789	0.0232	43	23232	McShane 1991
5.5–6.5 months (8–10 mm)	0.4/month	0.67	0.000850	1177	0.0381	26	38086	McShane 1991
0.5–1.5 years (10–35 mm)	0.91/year	0.403	0.000342	2920	0.0568	18	56844	Day & Leorke 1986
1.5–2.5 years (35–60 mm)	0.81/year	0.445	0.000152	6563	0.1411	7.1	141053	Day & Leorke 1986
2.5–3.5 years (60–85 mm)	0.42/year	0.67	0.000102	9795	0.3170	3.2	316973	Shepherd 1992, based on Shepherd & Hearn 1983
3.5–4.5 years (85–105 mm)	0.3/year	0.74	0.0000755	13236	0.4731	2.1	473095	Derived from data in Fig. 7
4.5–5.5 years (105–115 mm)	0.25/year	0.78	0.0000589	16970	0.6393	1.6	639317	Derived from data in Fig. 7
5.5–6.5 years (115–120 mm)	0.20/year	0.82	0.0000483	20695	0.8196	1.2	819638	Derived from data in Fig. 7

lion 2-mm postlarvae, needed to raise sustainable fisheries yields by 300 t, would be cost prohibitive using current commercial hatchery technology. Although the scale of such seed production dwarfs that of existing commercial hatcheries in southern Australia, it is nevertheless considered to be practicable and affordable provided much more space, labor and cost efficient hatchery and nursery technologies, developed in this project, are used. Using such technology would entail the reproductive conditioning and induced spawning of 3,200 captive female broodstock. Again, the most critical limitation of this seeding option is uncertainty surrounding the fact that it has not been experimentally trialed in the field let alone on a commercially significant scale.

4. *8-mm juveniles* ("buttons"). An estimated yield per recruit of 1 harvestable *H. rubra* per 26 "buttons" is based on mortality data of wild *H. rubra* data presented in Table 2. Production of the 26 million buttons required would entail the reproductive conditioning and induced spawning of 2,600 captive female broodstock (Table 3). As with the previous two alternative seed classes, this relatively large broodstock requirement is considered both practicable and affordable as is seed production using new high efficiency hatchery and nursery technology developed during this project. However unlike week-old postlarvae and 2-mm postlarvae, commercial-scale low density dispersed seeding methods for this class of seed have been fully developed and very extensively trialed in the field over the past 5 y.
5. *40-mm juveniles*. An estimated yield per recruit of 1 harvestable *H. rubra* per 15 seed comprising 40 mm juveniles, is based of mortality data of wild *H. rubra* data presented in Table 2. Production of the 15 million required juveniles would entail the reproductive conditioning and induced spawning of 2,900 captive female broodstock (Table 3). As with the previous three alternative seed classes, this broodstock requirement, though relatively large is considered both practicable and affordable. The extremely large seed production requirement of 15 million 40 mm juveniles equates to 150 t live weight, which is likely to be cost prohibitive being about twice the annual output of Australia's largest producing farm in 2004/5 (J. Hall, King Island Abalone P/L, pers. comm.). Further factors mitigating against these large seed are daunting logistical difficulties of handling, transporting and dispersion in the field.

Information provided in Table 3 summarizes and compares key logistical, technical and cost issues in producing and deploying the five alternative size/age classes of seed. It also compares utility of producing each size/age class using either conventional single annual batch hatchery production with much more efficient technology developed during this project.

Predictions regarding use of button size juveniles as seed are particularly encouraging in that the potential margin for profit forecast is so handsome that break-even enhancement would still occur even if survival of hatchery-produced seed fell as low as 10% that of wild counterparts. Alternatively, if average survival rates of say 25% to 50% of comparable wild stock are achievable, profitable seeding of depleted populations of abalone could still be attained at much smaller scales of operation than applied to this model, which assumes 100% parity with survival of wild counterparts.

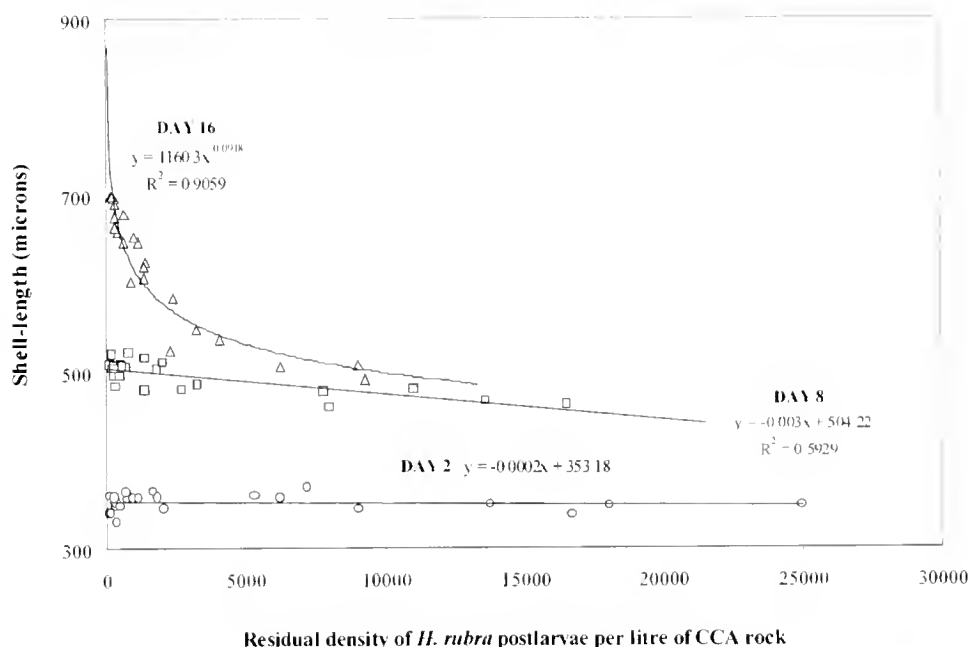


Figure 10. Relationship between residual density and growth of *H. rubra* postlarvae seeded onto natural CCA settlement substrates after 2, 8 and 16 days (Heasman et al. 2004).

The model also predicts that seeding of larvae is likely to be unprofitable and also casts doubt on the economic viability of large-scale seeding of natural reef using advanced (18-mo-old, 40-mm) juvenile *H. rubra*. This is not to say that cost-effective seeding of more advanced juveniles cannot be achieved under reduced competition for food and shelter from competitors and/or reduced predation. For example, R. Day (pers. comm.) reported 50% survival of green lip abalone (*H. laevis*) 1 year after being seeded as 28 mm SL juveniles onto reefs constructed from natural reef rocks in shallow, high food flux (drift seaweed) sea-grass beds.

#### RECENT DEVELOPMENTS

Continuing research of cost-effective technology for enhancing the *H. rubra* fishery in NSW and elsewhere in southern Australia is being pursued under a 12-mo extension of this project. Continuing research towards improving postrelease survival is now being undertaken by A. Underwood and G. Chapman (Sydney University). All field experimentation has been confined to the Port Stephens area because stock translocation limitations imposed by an outbreak of the disease *Perkinsus* that devastated the region's *H. rubra* stocks by an estimated 90% between 2000 and 2002 (Daly 2004). This research is further addressing a number of factors likely to influence postrelease survival that includes evaluation of:

- clustered and dispersed seeding of button size (5–15 mm) juveniles,
- size of juveniles within the button size range on postrelease survival,
- urchin shadows or similar devices for reducing early postrelease mortality,
- multiple low-density seeding of sites and single higher density seeding, and
- natural and artificial diets on postrelease performance of seeded juveniles.

Complementary research being undertaken by NSW Fisheries includes development of a novel method of achieving low density dispersed seeding. This has involved the design and manufacture of small, predator-protective release capsules that accommodate only 10–20 button size seed. These miniaturized deployment devices can be broadcast over entire juvenile habitats of depleted reefs during daylight hours from surface craft, thereby negating costly deployment by divers. A key design feature of the capsules is that they pack together to form complete level platforms. This feature together with the use of intensive light, encourages in excess of 95% of the "buttons" to self-load into the capsules

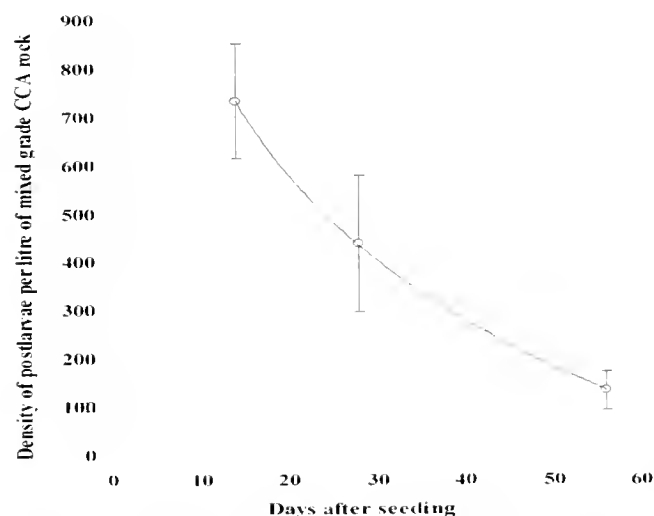


Figure 11. Residual density of *H. rubra* postlarvae per liter of CCA rock after 56 days. Bars are  $\pm SE$ ,  $n = 4$  (Heasman et al. 2004). Note: 100 postlarvae per liter of CCA rock represents 1000 per  $m^2$  of surface area.

TABLE 3.  
Bioeconomics model summarizing requirements and associated benefits and costs of producing and deploying five age/size classes of *H. rubra* aimed at increasing the sustainable commercial catch in NSW by 300 tonnes (1 million  $\times$  120 mm abalone).

Age & size of seed	Estimated Number of Hatchery			Estimated Number of Brood-Stock Spawners Needed to Yield 1 Million Legal Abalone Valued at ~\$15 M (Assumes Same Survival Rates as Equivalent Wild Stock)	Estimated Annual Operating Costs Using Conventional Hatchery Production and Seeding Techniques Plus General Comments	Estimated Annual Operating Costs Using Production and Seeding Techniques Developed in This Study NB Estimated Capital Cost of Hatchery = \$1 Million
	Assumed d Number of Seed Needed to Yield 1 Harvestable Abalone Averaging 120 mm and 300 g (from Table 2)	Broodstock of this Age/Size Needed to Yield 1 Million Legal Abalone	Legal Abalone Valued at ~\$15 M			
Competent larvae	20,000	20 Billion	33,000	Assumed fecundity of 1.5 M eggs/spawner and 40% yield of competent larvae from eggs = 0.6 M/spawner $\leq 3,300$	Logistical problems, of huge numbers of broodstock needed and deployment of larvae, appear insurmountable	Logistical problems associated with huge numbers of broodstock and deployment of larvae appear insurmountable
1 wk old postlarvae (0.4 mm)	2,000	$\leq 2$ Billion	3200	Assumed 50% mean yield of PL's from larvae = 0.3 M/spawner	Conventional hatchery and nursery technology is inappropriate	Promising option but technology of handling and deployment lacking
2 months ex plate (2 mm/0.002 g)	157	157 Million	3200	Assumed 20% yield to this age from 1 week post set = 0.06 M/spawner	Prohibitively costly (\$6.28 M) based on current commercial prices of 2 cents per mm = 4 cents per spat	\$0.5 M (see Table 4) Promising option but technology of handling and deployment lacking
6 month old juveniles (8 mm/0.1 g)	26	26 Million	2600	Assumed 10% yield to this age from 1 week post set = 0.03 M/spawner	Prohibitively costly (\$4.16 M) based on 2 cents per mm = 16 cents per spat	\$0.665 M (see Table 4)
21 months old juveniles (40 mm/10 g)	15	15 Million	2900	Assumed 9% yield to this age from 1 week post set = 0.027 M/spawner	Prohibitive: facility would need to be twice as big as Australia's largest farm i.e. biomass of seed = 150 tonnes/year	Prohibitive facility would need to be twice as big as Australia's largest existing farm i.e. biomass of seed = 150 tonnes/year

TABLE 4.

Estimated annual operating budgets for hatchery production and seeding of 157 million 2 mm *H. rubra* postlarvae and 26 million 8 mm juveniles. Note: these budgets incorporate new highly efficient hatchery and nursery technologies developed in this study but do not include depreciation on plant and equipment.

Salaries (Including 30% On-Costs)	2 mm Postlarvae \$	8 mm Juveniles \$
Seed Production		
Full time manager	100,000	100,000
Full time senior fisheries technician	80,000	80,000
Full time fisheries technician(s)	60,000	120,000
Seed deployment and monitoring		
Casual assistants	30,000	60,000
Sub Total	270,000	360,000
Travel & Accommodation		
20 trips for 3 days by 3 staff @ \$120/d/person	21,600	21,600
Operating costs		
Power	60,000	80,000
Repairs & Maintenance	30,000	40,000
Vehicle operating	12,000	12,000
Post freight & packaging	5,000	5,000
Air fills	3,000	3,000
Telephone/email/www	3,000	3,000
Boat operating	10,000	10,000
Consumables	10,000	10,000
Chemicals/pharmaceuticals	3,000	3,000
Insurances	1,000	1,000
Food	1,000	10,000
Permits	1,000	1,000
Sub Total	139,000	178,000
Interest on \$1 m or \$1.5 m for capital works loans at 7% pa	70,000	105,000
Total	500,600	664,600

(Heasman, unpublished data). Once loaded, the "buttons" can be maintained within the capsules in good health for several days of storage awaiting either more favorable sea and weather conditions or off-road transportation to sites up to several hundred kilometers from the hatchery. The latest advance in this technology has been the development of two alternative environmentally friendly forms of the capsule. One is manufactured from a specially developed biodegradable resin and the other of aluminum that will corrode away in seawater within a year.

In late July and early August 2005, almost 60,000 button size (average SL 11 mm) juveniles were stocked into aluminum dispersion release devices. Approximately 560 devices containing an average of 17 "buttons" were used to seed each of six 1,000 m<sup>2</sup> sites marked by surface buoys. All six seeded and an additional six unseeded control sites were located along an 8 km section of coast on the southern side of the Tomaree Peninsula at Port Stephens. All sites were considered typical juvenile habitat comprising a high component of crustose coralline algae coated boulders, immediately adjacent to locations identified by local commercial fishermen to have consistently supported good catches prior to *Perkinsus* epidemics that decimated these stocks between 2000 and 2002. Broadcast seeding of the capsules from a 10-m vessel required only a few minutes per site. Aims of this long term experiment are to evaluate the commercial utility of dispersed

seeding of depleted stocks and to assess genetic impacts of such releases.

Possible deleterious genetic consequences of seeding large numbers of juveniles produced in the hatchery from relatively small number of parents have also been addressed in the current project through the development and assessment of triploid *H. rubra*. Chemical induction techniques yielding almost 100% stable triploids were developed. These triploids were subsequently shown to be reproductively sterile and although no whole weight growth advantage could be demonstrated in triploids up to 37 mo old and 70 mm SL, yield of saleable flesh was 20% greater than in full sib diploids (Lui 2005). Furthermore, one particular treatment used to induce triploidy was found to have inadvertently produced significant numbers of tetraploid individuals paving the way for future research to produce 100% functionally sterile triploid progeny by fertilizing eggs from diploid females with sperm of a tetraploid males (Liu et al. 2004).

## CONCLUSION

Considerable progress towards cost effective enhancement of the NSW abalone fishery has been made through reducing costs of production and transportation of *H. rubra* seed and from the identification of button size, 6–9-mo-old juveniles as the most appropriate class of seed for enhancing depleted subpopulations. The detection of a major apparent flaw in the use of clustered deployment of these juveniles and development of low density dispersed seeding technology is also a significant step forward. This will hopefully enable seeding to be aligned with surface grazing based on carrying capacity limitations of CCA rock habitats in relation to juveniles up to at least 12 mo old and 25 mm SL.

Nevertheless, many other major questions and issues remain to be addressed. Foremost is the issue of matching seeding operations with inherent capacities of individual depleted reefs to sustainably yield higher quantities of marketable 4+-year-old adults. Division of the NSW abalone fishery into six geographical management zones (Fig. 12) reflects a very steep south to north gradient of decreasing abundance of *H. rubra*. Almost 80% of TACC quotas are taken on average from zones 4–6 that collectively fall within 150 km of the Victorian border (Cape Howe). At the opposite extreme, catches from zone 1, that stretches 600 km north from Jervis Bay to the Queensland border, have fallen from around 20% of total commercial landings in the mid 1980s to only about 5% over the recent years (Anon. 2002). The relative importance of such interactive factors as recruitment, availability of food, primarily drift seaweed (Shepherd & Hearn 1983), competition for food and shelter and differential mortality factors, including natural predation and fishing pressure, plus disease (especially *Perkinsus*) on this changing pattern of abundance, is not well understood.

Higher sea temperatures in the north are undoubtedly a greater constraint to full development of gonads and hence to successful spawning and recruitment in some years. These processes require at least 1,200 degree days at moderate temperatures in the range 8°C to 18°C (Grubert & Ritar 2003). Likewise, losses of up to 90% of stocks in the Port Stephens area of zone 1 in 2000 and 2002, attributed to stress-induced susceptibility of *H. rubra* to the disease *Perkinsus*, could, together with impaired breeding, be linked to increased water temperatures. This condition has also been suggested by Shepherd et al. (1998) as a possible cause of the decline of another abalone fishery.

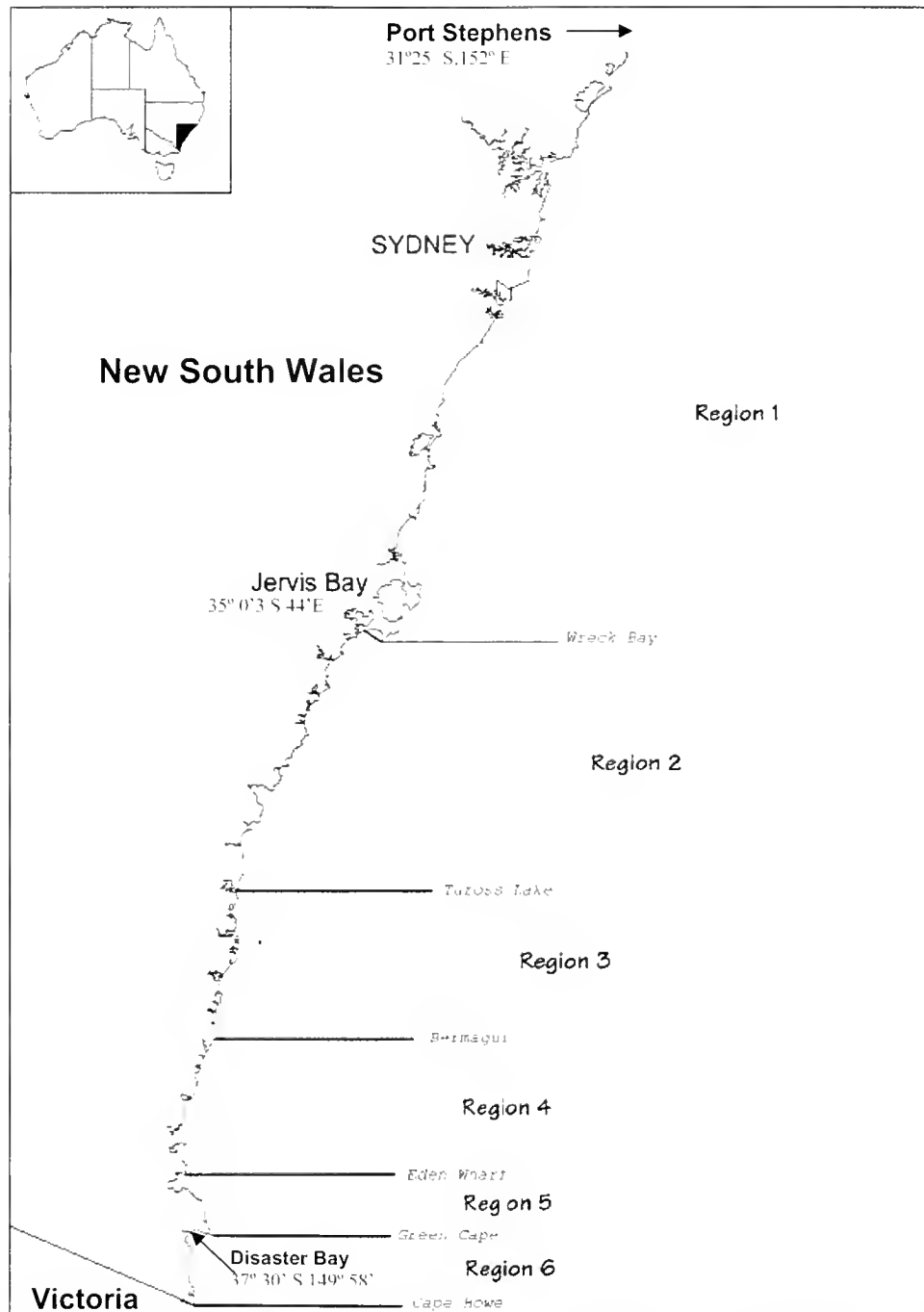


Figure 12. Map of New South Wales coastline from Port Stephens south to the Victorian border, showing abalone fishery zones.

Future development of tetraploid *H. rubra* offers the prospect of using functionally sterile 100% triploid seed to enhance depleted reefs. This could in turn enable "put and take" ranching in NSW, capitalizing on large-scale, cost efficient, centralized production of seed. Home ranges of *H. rubra* and natural recruitment in relation to parent stock, have both been shown to be limited to scales of several hundred meters in *H. rubra*. Likewise, productivity of particular areas of reef can vary greatly over distances of hundreds or even tens of meters. Effective management protocols therefore need to be tailored to accommodate these fine spatial scales.

In addition to a put-and-take ranching of sterile triploid seed,

there seems to be good scope for rebuilding depleted natural populations of *H. rubra* in NSW. However, for that strategy, seed will need to be produced in hatcheries from genetically matched parent stock in sufficient numbers to safeguard the integrity of natural gene pools, including preservation of rare or unique alleles. Rebuilding standing stocks of *H. rubra* to high levels at which self sustaining natural recruitment can be restored, will probably require integration of seeding with new and innovative fisheries management initiatives. Such initiatives will need to address apparent imbalances that have developed between *H. rubra* and several of its major competitors, especially the black urchin *C. rodgersii* and other large and common grazing gastropods, especially

*Turbo torquatus* (Gmelin, 1791), *Turbo militaris* (Reeve, 1848) and *Astralium tentoriformis* (Jonas, 1845). This in turn can only be achieved through the comanagement of these competing species (Andrews et al. 1998). In contrast to *H. rubra*, black urchins and the other common gastropods cited earlier have not to date been subjected to substantial levels of fishing pressure in NSW. It is also likely that efficient reseeding protocols will need to be aligned to the patchy distribution of legal size adults. In practice this will entail targeted reseeding of juvenile habitat that lies adjacent to high yielding patches of reef. Such "hot spots" for abalone and drift seaweed are well known to experienced commercial divers and are commonly characterized by fast growing abalone with elongated shells (Worthington & Andrew 1997).

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## EXPRESSED SEQUENCE TAG ANALYSIS OF GENES EXPRESSED DURING DEVELOPMENT OF THE TROPICAL ABALONE *HALIOTIS ASININA*

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**ABSTRACT** The tropical abalone, *Haliotis asinina*, is an ideal species to investigate the molecular mechanisms that control development, growth, reproduction and shell formation in all cultured haliotids. Here we describe the analysis of 232 expressed sequence tags (EST) obtained from a developmental *H. asinina* cDNA library intended for future microarray studies. From this data set we identified 183 unique gene clusters. Of these, 90 clusters showed significant homology with sequences lodged in GenBank, ranging in function from general housekeeping to signal transduction, gene regulation and cell-cell communication. Seventy-one clusters possessed completely novel ORFs greater than 50 codons in length, highlighting the paucity of sequence data from molluscs and other lophotrochozoans. This study of developmental gene expression in *H. asinina* provides the foundation for further detailed analyses of abalone growth, development and reproduction.

**KEY WORDS:** abalone, EST, Lophotrochozoan, development, *Haliotis asinina*

### INTRODUCTION

Abalone (*Haliotis* spp.) are a particularly attractive aquaculture species because of their high demand, high market value and limited supply (Gordon & Cook 2001, Oakes & Ponte 1996). Whereas temperate abalone form the majority of abalone exports worldwide, there is a growing demand for tropical species (Jarayabhand & Paphavasit 1996). The tropical Indo-Pacific, including Australia, is well positioned to capture and further develop this market through the aquaculture of the rapidly growing tropical species *Haliotis asinina* Linnaeus. This species reaches marketable size far quicker than the temperate species (Capinpin & Corre 1996, Capinpin et al. 1999) and is ideal for the new "cocktail"-sized abalone market (Nateewathana & Hylleberg 1986, Singhagraiwan & Doi 1993).

We have been using the tropical abalone to address questions pertaining to development (e.g., Giusti et al. 2000, Hinman & Degnan 2002, Hinman et al. 2003, O'Brien & Degnan 2002a, 2002b, O'Brien & Degnan 2003, Jackson et al. 2005) and as a model to investigate the molecular basis of commercially important developmental and physiological traits (Jackson et al. 2005, Selvamani et al. 2001, Selvamani et al. 2000). With a frequent and predictable spawning cycle (Counihan et al. 2001, Jebreen et al. 2000) and established techniques for culturing to sexual reproduction (Jackson et al. 2001), gene expression can be easily studied at any stage of the life cycle. As a member of the Lophotrochozoa, the least investigated bilaterian superphylum (Adoutte et al. 2000), analyses of *H. asinina* development also contributes to a general understanding of metazoan evolution and development.

As a prelude to the sequencing of the human genome, over 170,000 ESTs were sequenced in an attempt to identify new genes and their expression patterns (Adams et al. 1995). This approach has been applied to a wide range of species, permitting the identification of homologous genes from organisms not previously studied (see [http://www.ncbi.nlm.nih.gov/dbEST/dbEST\\_summary.html](http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html) for a comprehensive list). Included in this public database are a number of marine invertebrates: the ascidians, *Ciona intestinalis* (684,319 ESTs), *Halocynthia roretzi* (4192 ESTs) and *Polyandrocarpa misakiensis* (465 ESTs), the lancelet *Branchiostoma floridae* (277,538), the sea urchin *Strongylocen-*

*trotus purpuratus* (130,988 ESTs), the oyster *Crassostrea virginica* (9018 ESTs) and the prawn *Penaeus monodon* (1611 ESTs) (dbEST release July 22, 2005). However there is a significant bias in this database towards vertebrates, model organisms and terrestrial species of commercial value. Here we describe a small-scale EST analysis of cDNAs obtained from a cDNA library constructed from a range of developmental stages of the tropical abalone *H. asinina*.

### MATERIALS AND METHODS

#### RNA Extraction and Library Construction

Total RNA was extracted according to the method of Chomczynski and Sacchi (1987) from the following developmental stages: egg, gastrula, early trochophore (13 h post fertilization; hpf), mid torsion (18 hpf), early veliger (24 hpf), mid veliger (40 hpf), early competent veliger (72 hpf), late competent veliger (134 hpf), 1 day post metamorphosis and 5 days post metamorphosis. All RNAs were quantified spectrophotometrically and quality-assessed by inspection of samples that had been electrophoresed through a 1% formaldehyde gel (Sambrook & Russell 2001). To maximize gene representation in the cDNA library 200 ng of total RNA from each stage was combined for library construction using the directional Clontech SMART cDNA library synthesis kit. Following the manufacturers instructions, double stranded cDNA was PCR amplified for 23 cycles prior to cloning. Five-thousand plaques were individually hand picked following methods outlined by Sambrook and Russell (2001) for microarray printing. Of these 288 plaques were PCR amplified using the  $\lambda$ Triple X forward (CTCGGGAAGCGCGCCATTGTGTTGGT) and reverse (TAATACGACTCACTATAGGGCGAATTGGCC) primers. Successful PCR reactions were subsequently purified with the Millipore multiscreen PCR clean up kit. Two-hundred and thirty-two clones were single-pass sequenced using the  $\lambda$ Triple X forward primer and ABI Big Dye terminator mix (version 2) following standard procedures (2001).

#### Sequence Analysis

Sequencing chromatograms were assessed manually using 4Peaks (v1.5) and were truncated to eliminate ambiguous terminal base calls and vector sequence. BLASTx and BLASTn homology searches were conducted against the NCBI nr databases using the

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default conditions (low complexity filter on, an expect value of 10 and the BLOSUM62 matrix with existence 11 and extension 1 gap costs). Sequences that returned BLAST matches with an E value of  $1e^{-06}$  or less were considered significant, and allowed a functional classification to be loosely made following the scheme of Lee et al. (1999). To assess sequence redundancy or "uniqueness," sequences were clustered using ClustalW (<http://www.ebi.ac.uk/clustalw/index.html?>), (Chenna et al. 2003). The integrity of each cluster was assessed manually by visually inspecting cluster alignments for overlap quality. Where cluster members were found to share more than 95% identity the cluster was usually maintained.

ESTs were also assessed for the presence of a putative poly (A) tail and an associated polyadenylation signal based on the classification of Beaudoin et al. (2000). Those sequences that did not return any significant BLAST result were assessed for the presence of an open reading frame (ORF) using the European Bioinformatics Institute Transeq server (<http://www.ebi.ac.uk/emboss/transeq/>). Only ORFs longer than 50 amino acid residues were accepted (Lee et al. 1999, Lehninger et al. 1993).

## RESULTS

The *Haliotis asinina* developmental cDNA library contained  $5 \times 10^7$  pfu/mL, of which approximately 93% were recombinant clones. These had an average insert size of  $1044 \pm 411$  bp. Clustering of the 232 sequences revealed that 157 sequences occurred only once, 16 sequences occurred twice, 3 sequences occurred

three times, 3 sequences occurred four times, 3 sequences occurred 5 times and 1 sequence occurred 7 times, producing 183 unique clusters (Table 1).

Homology searches using the BLASTx and BLASTn algorithms were conducted for all sequences. 90 clusters returned a significant BLAST result and are grouped according to function in Table 2. Sixty-eight clusters fell into the category of genes used by many cell types, 8 clusters showed homology with genes involved in cell-cell communication, 3 clusters showed homology with transcription factors, and they are likely to play important developmental or biosynthetic roles in *H. asinina*. Eleven clusters showed significant homology with an undescribed ORF and could not be classified (Fig. 1; Table 1). These EST sequences have been submitted to GenBank under accession numbers DY402832–DY403158.

### Polyadenylation Signal and ORF Analysis

Of the 183 unique clusters, 81 were completely sequenced in one pass and possessed a poly (A) -rich region at the 3' end of the sequence. Of these 81, 54 possessed a putative polyadenylation signal a mean distance of 17 nucleotides upstream of the poly (A) tail. The frequency of polyadenylation signals alternative to the predominant AATAAA signal was remarkably similar to that of human signals reported by Beaudoin et al. (2000) (Table 3). We also found that out of all ESTs with a polyadenylation signal, 5 (2.1%) had dual polyadenylation signals an appropriate distance

TABLE 1.  
Summary of ESTs grouped by category and BLAST results.

Code Class		No. of Clusters	No. of Clones
A. Functions that many kinds of cells use			
AI	Transport and binding proteins for ions and other small molecules	1	1
AII	RNA processing, polymerising, splicing and binding proteins	4	4
AIII	Cell replication, histones, cyclins and allied kinases, DNA polymerases	4	5
AIV	Cytoskeleton and membrane proteins, cellular organisers	7	8
AV	Protein synthesis cofactors, ribosomal proteins, rRNAs	29	47
AVI	Intermediary metabolism and catabolism enzymes	7	7
AVII	Stress response, detoxification, and cell defense proteins	8	18
AVIII	Protein degradation and processing, proteases	3	3
AIX	Transportation and binding proteins for proteins and other macromolecules	3	4
AX	Proteins involved in motility including muscle components	2	1
	Total	68	98
B. Cell-cell communication			
BI	Signalling receptors and ligands	4	4
BII	Intracellular signaling molecules, including kinases and signal intermediates	3	3
BIII	Extracellular matrix proteins and cell adhesion	1	1
	Total	8	8
C. Transcription factors and other regulatory proteins			
CI	Sequence-specific DNA-binding proteins	3	3
CII	Non-DNA binding proteins that have positive or negative roles	0	0
CII	Chromatin proteins other than AIII with regulatory function	0	0
	Total	3	3
D. Not enough information to classify			
DI	Not enough information to classify	11	11
DII	No significant similarities to known proteins	93	112
	Total	104	123
	Overall total	183	232
	Single copy sequences	157	
	Total no. of ESTs represented more than once	75	
	Total no. of redundant sequences	49	
	Total number of unique sequences (total number of clusters)	183	
	Clusters returning significant BLAST match	90	
	Clusters without any GenBank homologue	93	

TABLE 2.  
Significant BLAST results grouped according to function.

Clone	e Value	Accession	Gene Name	Organism	Scientific Name
AI. Transport and binding proteins for ions and other small molecules					
2G2	5e-14	CAG04534	GM2 ganglioside activator*	Pufferfish	<i>T. nigroviridis</i>
AII. RNA processing, polymerising, splicing and binding proteins and enzymes					
1H1	1e-45	AAH20773	Exosome component 8	Human	<i>H. sapiens</i>
2C9	3e-18	XP_391989	Similar to thickveins	Honey bee	<i>A. mellifera</i>
15C8	6e-27	CAF93482	G patch domain*	Pufferfish	<i>T. nigroviridis</i>
15D4	2e-30	IFS2_ID	RNA polymerase II elongation factor*	Human	<i>H. sapiens</i>
AIII. Cell replication, histones, DNA polymerases, topoisomerases, DNA modification					
2B6	1e-48	P08991	Histone H2A variant	Urchin	<i>S. purpuratus</i>
15B4	6e-44	AAC47489	Enhancer of rudimentary	Mosquito	<i>A. aegypti</i>
15G2	2e-21	CAA28177	Histone H1-beta*	Painted urchin	<i>L. pictus</i>
2C7	4e-13	XM_315130	Chief histone H3 (2)	Mosquito	<i>A. gambiae</i>
AIV. Cytoskeleton and membrane proteins, cellular organisers					
1A11	4e-38	NP_990573	Low density lipoprotein	Chicken	<i>G. gallus</i>
1B5	2e-49	CAF21863	Gelsolin	Sponge	<i>S. ficus</i>
1D7	1e-108	U30467	Alpha-tubulin	Spoon worm	<i>U. caupo</i>
1H7	3e-45	CAD91425	Actin related protein	Oyster	<i>C. gigas</i>
2G1	2e-15	CAG07401	Low density lipoprotein*	Pufferfish	<i>T. nigroviridis</i>
15D9	2e-82	AY026071	Beta-tubulin	Choanoflagellate	<i>M. brevicollis</i>
15D10	2e-43	AF510206	Beta-tubulin	Ciliate	<i>O. longa</i>
AV. Protein synthesis cofactors, tRNA synthetase, ribosomal proteins, rRNAs					
15H3	e-147	AY163259	16S ribosomal RNA (7)	Tropical abalone	<i>H. asinina</i>
2A7	4e-42	AAN05608	Ribosomal protein L26 (2)	Scallop	<i>A. irradians</i>
15E5	3e-13	U51989	16S ribosomal RNA gene (2)	Abalone	<i>H. diversicolor</i>
2H5	e-105	AY163259	16S ribosomal RNA (4)	Tropical abalone	<i>H. asinina</i>
2A5	1e-60	AY145418	28S ribosomal RNA (2)	Abalone	<i>H. discus</i>
15F5	1e-52	AY588938	Mitochondrion, complete genome (2)	Black lip abalone	<i>H. rubra</i>
15C9	8e-97	AY163259	16S ribosomal RNA (2)	Tropical abalone	<i>H. asinina</i>
1B10	3e-48	AAP33157	Beta-NAC-like protein	Termite	<i>R. flavipes</i>
1E3	4e-25	AAC15656	60S ribosomal protein P2	Chiton	<i>C. stelleri</i>
1E10	1e-22	Z18289	16S rRNA gene	Red alga	<i>P. palmata</i>
1G4	5e-34	AF120512	18S ribosomal RNA	Slit shell	<i>S. confusa</i>
1G10	2e-98	AY588938	Mitochondrion, complete genome	Black lip abalone	<i>H. rubra</i>
2A12	5e-30	X80345	28S ribosomal RNA	Aquatic fungus	<i>H. catenoides</i>
2B3	2e-11	EAA59486	Eukaryotic initiation factor*	Fungi	<i>A. nidulans</i>
2E12	4e-98	AY145418	28S ribosomal RNA	Abalone	<i>H. discus</i>
2F7	e-114	AY145418	28S ribosomal RNA	Abalone	<i>H. discus</i>
2H12	2e-42	M98364	Ribosomal RNA	Ciliate	<i>Coleps sp.</i>
15A3	4e-57	AAA70102	40S ribosomal protein S24	Slime mould	<i>D. discoideum</i>
15A4	9e-12	CAG13823	Mitochondrial ribosomal protein L27*	Pufferfish	<i>T. nigroviridis</i>
15A7	9e-35	EAA01025	60S ribosomal protein L6*	Mosquito	<i>A. gambiae</i>
15B1	8e-25	AY691376	23S ribosomal RNA	Bacteria	<i>Burkholderia sp.</i>
15B2	9e-40	AAM94271	Ribosomal protein S2	Scallop	<i>C. farrieri</i>
15B7	1e-74	AY588938	Mitochondrion, complete genome	Black lip abalone	<i>H. rubra</i>
15B12	4e-24	BX950290	Ribosomal protein L38*	Chicken	<i>G. gallus</i>
15E4	4e-66	AAN05595	Ribosomal protein S8	Scallop	<i>A. irradians</i>
15E8	6e-29	AAP21827	Ribosomal protein S29	Amphioxus	<i>B. tsingtaunense</i>
15F10	2e-32	AAH86809	Translation initiation factor*	Zebrafish	<i>D. rerio</i>
15G9	6e-40	CAG02712	Elongation Factor Tu*	Pufferfish	<i>T. nigroviridis</i>
15G10	5e-70	AB003720	Elongation factor 1	Turbo	<i>B. cornutus</i>
AVI. Intermediary metabolism and catabolism enzymes					
2D3	6e-17	AY431429	Carboxypeptidase A	Mosquito	<i>A. aegypti</i>
1C1	2e-26	NP_918077	Putative carboxymethylglutaminylase	Rice	<i>O. sativa</i>
1F4	1e-38	XP_316948	Nudix*	Mosquito	<i>A. gambiae</i>
1F10	1e-76	CAE70680	Isocitrate dehydrogenase*	Nematode	<i>C. elegans</i>
1H6	3e-27	XP_308198	Pancreatic lipase*	Mosquito	<i>A. gambiae</i>
15C3	2e-20	CAA93088	Glutathione S-transferase*	Nematode	<i>C. elegans</i>
15G3	9e-11	EAL31332	Maleylacetoacetate isomerase*	Fly	<i>D. pseudoobscura</i>

continued on next page

TABLE 2.  
continued

Clone	e Value	Accession	Gene Name	Organism	Scientific Name
AVII. Stress response, detoxification, and cell defence proteins					
15E2	2e-53	YP_026076	Cytochrome b (3)	Black lip abalone	<i>H. rubra</i>
2E5	1e-37	AAR11781	Heat shock protein 90 (2)	Scallop	<i>C. farreri</i>
2E10	1e-15	AAK56498	Putative metallothionein (2)	Perrinkle	<i>L. litorea</i>
15F3	4e-09	AAM20842	SARP-19 precursor (5)	Periwinkle	<i>L. litorea</i>
1F9	3e-27	CAF99069	Ubiquinol-cytochrome C*	Fish	<i>T. nigroviridis</i>
1G9	1e-14	ZP_00316378	Fucose binding lectin*	Bacteria	<i>M. degradans</i>
2H11	5e-11	EAA03739	UQCRH protein*	Mosquito	<i>A. gambiae</i>
15B9	1e-25	AAP31550	HSB1-like protein	Fruit fly	<i>D. melanogaster</i>
AVIII. Protein degradation and processing, proteases					
2C2	3e-26	AAH87343	Proteasome 26S subunit*	African clawed frog	<i>X. laevis</i>
2C4	3e-52	AAH42820	PSMAS protein	Human	<i>H. sapiens</i>
2E11	2e-29	XP_394993	Proteasome subunit beta	Honey bee	<i>A. mellifera</i>
AIX. Transportation and binding proteins for proteins and other macromolecules					
1B12	1e-67	AAT44866	Translocon-associated protein (2)	Amphioxus	<i>B. tsingtaunense</i>
1B9	6e-14	XP_518767	Heme binding protein*	Chimpanzee	<i>P. troglodytes</i>
15F6	3e-24	AAH54875	TIMM19 protein	Human	<i>H. sapiens</i>
AX. Proteins involved in motility including muscle components					
15F12	8e-50	XP_537691	Dynem light chain-2	Dog	<i>C. familiaris</i>
2F9	1e-10	EAL32148	Myosin II*	Fly	<i>D. miranda</i>
BI. Signalling receptors including cytokine and hormone receptors, and signalling ligands					
1A12	2e-13	F44840	FMRF amide	Pond snail	<i>L. stagnalis</i>
1C5	4e-7	AF076823	Lysm precursor	Red abalone	<i>H. rufescens</i>
1E11	1e-14	NP_001008891	Signal sequence receptor	Rat	<i>R. norvegicus</i>
2F12	4e-37	AAT00460	Endozepine	Carp	<i>C. carpio</i>
BII. Intracellular signal transduction pathway molecules, including kinases and signal intermediates					
15D12	4e-12	EAL51743	Protein kinase	Entamoeba	<i>E. histolytica</i>
15E1	3e-14	AAD34418	Calmodulin mutant	Synthetic construct	x
15F11	1e-24	AAO62074	Rho-GTPase-activating protein	Mouse	<i>M. musculus</i>
BIII. Extracellular matrix proteins and cell adhesion					
2H9	8e-11	Q01528	Aggregation factor	Horseshoe crab	<i>L. polyphemus</i>
CI. Sequence-specific DNA-binding proteins					
2A9	7e-10	XP_470396	Putative pirin-like protein	Rice	<i>O. sativa</i>
2C10	2e-19	AAN31640	High mobility group protein 1	Snail	<i>B. glabrata</i>
2F1	5e-38	AAP97158	EVORF	Human	<i>H. sapiens</i>

\* Indicates gene names that do not belong to the most significant BLAST match for that EST. The presented gene name is taken from the next most significant BLAST match that allowed classification.

Numbers in bold parenthesis represent the total number of clones in that cluster.

upstream (both within 35 bp) of the poly A tail. This compares with 16.6% of human genes with dual polyadenylation signals reported by Beaudoin et al. (2000).

Of the 93 clusters that did not produce a significant BLAST result, 71 possessed ORFs equal to or longer than 50 codons (150 nucleotides), suggesting that they represent transcribed genes with no reported homologue.

## DISCUSSION

A decade ago Adams et al. (1995) established a set of stringent criteria for selection of cDNA libraries of adequate quality to be used in a large scale human EST analysis. These criteria included the following parameters: (1) less than 20% of the clones in library should have no insert or ribosomal and mitochondrial sequences; (2) the average insert lengths should be 1 kb or greater; (3) at least 50% of the cDNAs should encode novel genes and (4) no gene or

group of genes should dominate the distribution. These authors advocate the sequencing of 100–200 clones as an excellent means of assessing library quality based on these criteria. Here we have constructed a cDNA library from 10 distinct stages of development from the tropical abalone *Haliotis asinina*. Upon sequencing 232 randomly selected clones, we found that this library satisfies all of the criteria outlined by Adams et al. (1995). Searching the NCBI databases using the BLAST algorithms, 90 of the 183 clusters (49.1%) are significantly similar to previously described sequences.

As Lee et al. (1999) points out, one of the great advantages of an EST analysis of a novel cDNA library constructed from relatively poorly studied tissues is the generation of probes for "interesting" genes. This study has revealed a number of ESTs that deserve further investigation in terms of their temporal and spatial expression profiles, and the developmental and physiological roles

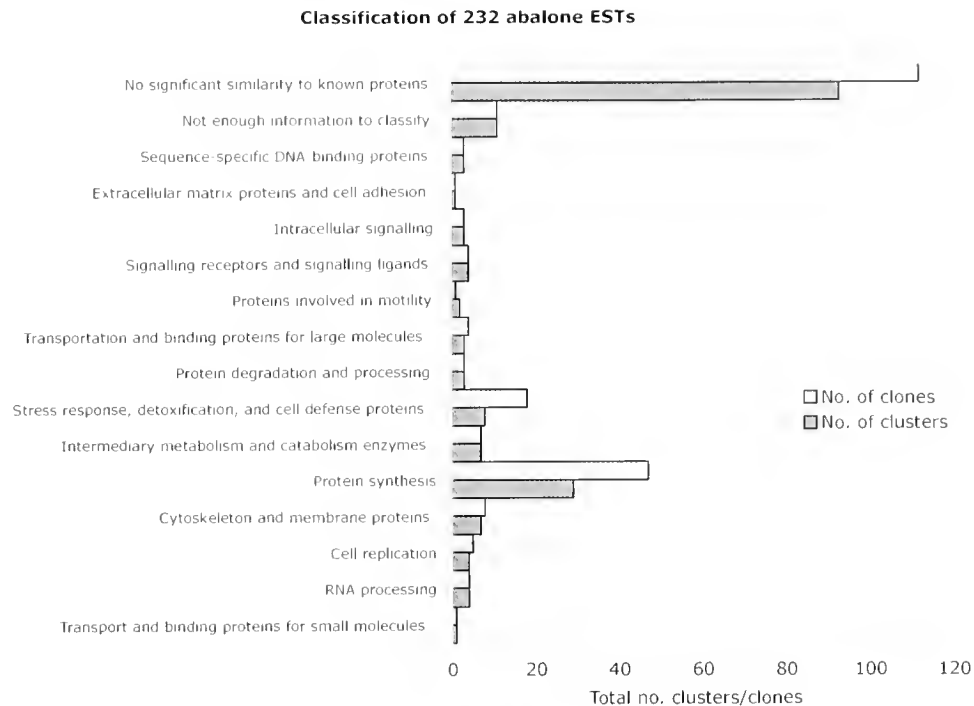


Figure 1. Prevalence of abalone ESTs grouped according to function.

that they play in the tropical abalone. For example, a significant proportion of the ESTs encode proteins that appear to be involved with stress response, detoxification, cell defense and innate immunity (Fig. 1). Analyses of differential gene expression in the as-

cidians *Boltenia villosa* and *Herdmania momus* during larval development and metamorphosis have revealed that a significant proportion of the expressed genes are involved with the innate immune system, with most of these being up-regulated during metamorphosis (Davidson & Swalla 2002, Woods et al. 2004). Components of the innate immune system may therefore be responsible for recognizing and responding to bacterial settlement cues associated with a suitable settlement environment. Bacterial biota associated with coralline algae may be responsible for induction of abalone settlement and metamorphosis (Bryan & Qian 1998, Roberts 2001).

Genes involved in cellular metabolism are also well represented in this EST set (7 clusters). Because larvae of *H. asinina* are lecithotrophic (nonfeeding), maternal energy reserves are utilized during larval development and metamorphosis. Genes encoding certain digestive enzymes however only appear to be activated during late larval development (Degnan et al. 1995, Spaulding & Morse 1991), whereas enzymes required for algal digestion are not detectable until postlarvae are significantly older (Takami et al. 1998). The proteases and metabolic enzymes identified here provide a platform from which to further our understanding of the processes that regulate and limit larval and juvenile growth.

An FMRF-encoding gene was also detected in this EST survey. In the gastropod mollusc *Lymnaea stagnalis*, expression of this gene is affected by infection of a schistosome parasite. This seems to be a strategy of the parasite to induce physiological (growth and reproduction) and behavioral changes in the host (Hoek et al. 1997). Among natural populations *H. asinina* can become infected by a digenae parasite that has been suggested to influence the antagonism between growth and reproduction cycles (Lucas et al. 2005), with infected animals effectively being castrated. The FMRF-neuropeptide identified here provides a means with which to study this phenomenon (Rice & Degnan, unpublished data).

Of the 90 unique clusters that returned significant BLAST

TABLE 3.

Prevalence of polyadenylation signals among abalone and human mRNA transcripts.

Polyadenylation Signal	Prevalence (%) and Location <sup>1</sup> in Abalone ESTs	Prevalence (%) and Location <sup>1</sup> in Human ESTs
AATAAA	(55.1) 15.3 ± 3.9	(58.2) 16 ± 4.7
ATTAAA	(12.3) 19.3 ± 7.9	(14.9) 17 ± 5.3
AGTAAA	(6.1) 16.7 ± 1.5	(2.7) 16 ± 5.9
TATAAA	(0.0)	(3.2) 18 ± 7.8
CATAAA	(2.0) 13.0 ± NA	(1.3) 17 ± 5.9
GATAAA	(2.0) 18 ± NA	(1.3) 18 ± 6.9
AATATA	(4.1) 18 ± 1.4	(1.7) 18 ± 6.9
AATACA	(2.0) 24 ± NA	(1.2) 18 ± 8.7
AATAGA	(2.0) 15 ± NA	(0.7) 18 ± 6.3
AAAAAG	(4.1) 22.5 ± 13.4	(0.8) 18 ± 8.9
ACTAAA	(2.0) 26 ± NA	(0.6) 17 ± 8.1
GACAAA <sup>2</sup>	(2.0) 21 ± NA	—
ATGAAA <sup>2</sup>	(4.1) 18 ± 2.8	—
AGAAAA <sup>2</sup>	(2.0) 16 ± NA	—
Total	100%	87%

<sup>1</sup> The location reported here is the position of the sixth nucleotide of the hexamer relative to the beginning of the poly A tail.

<sup>2</sup> These 3 hexamers were not reported by Beaudoin et al. (2000) but are included here as they only differ by one nucleotide from recognized polyadenylation signals, and were located within 30 nucleotides of the poly A rich 3' region, suggesting that they may be alternative abalone polyadenylation signals.

matches, 11 (12.2%) could not be assigned a function. This value is less than that reported by Adams et al. (1995) for a human EST study (24.8% of 266,714 ESTs), but much larger than 5.2% reported by Lee et al. (1999) for a sea urchin EST analysis, and 4.1% reported by Davidson and Swalla (2002) for an ascidian EST analysis. These figures reflect both the exponential increase in the number of annotated sequences housed in the NCBI database since 1995 (the 1995 Genbank database was 3.7% the size of the 2001 database), and the fact that *H. asinina* belongs to the poorly studied Lophotrochozoan clade in terms of sequence representation and annotation.

After an analysis of those ESTs that showed no homology with any sequences in the GenBank database, Lee et al. (1999) estimated that 65% to 80% of these sequences were genuine protein coding sequences that were simply too divergent to match anything in the largely mammalian GenBank database. This estimate was based on an analysis of observed versus expected ORF length within each EST. Briefly, randomly generated sequences equal in length to those ESTs analyzed were assessed for the presence of the longest ORF. These derived ORFs rarely exceeded 150 base pairs. Here we analyzed novel clusters (clusters that showed no homology with GenBank sequences) for the presence of ORFs greater than 150 nucleotides in length, in an effort to identify genuine coding transcripts that have no homologues within GenBank. Of the 93 novel clusters, 71 (76.3%) possessed an ORF in excess of 50 codons. This is similar to Lee et al.'s (1999) estimate of *Strongylocentrotus purpuratus* specific mRNAs of 65% to 80%.

Current developmental studies on *H. asinina* further support the notion of a high proportion of *H. asinina* specific genes (Jackson et al. 2005). Interestingly, studies on *C. elegans* and *Drosophila*, for which entire genomes are available, have revealed lower estimates of organism-specific coding sequences: 50% and 30% respectively of all recognized coding sequences (Harrison et al. 2002). If representative, this high proportion of novel coding mRNA transcripts for *H. asinina* further highlights the need for more studies of this kind to be conducted on the poorly studied Lophotrochozoan clade.

## CONCLUSION

This small EST project has demonstrated that the *H. asinina* library we have constructed consists of a diverse set of cDNAs that are of a large enough size to be useful in future microarray and gene characterization studies. It has also shown that during development, embryos and larvae of the tropical abalone express genes involved in a range of functions, ranging from cell defense and innate immunity to signal transduction and metabolism. The percentage of EST sequences that do not have homologues in the NCBI database highlights the need for further EST projects to be conducted on more diverse organisms, especially taxa belonging to the Lophotrochozoa.

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## DISEASES OF PEARL OYSTERS AND OTHER MOLLUSCS: A WESTERN AUSTRALIAN PERSPECTIVE

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**ABSTRACT** Mollusc culture, particularly the cultivation of pearl oysters, is an important component of the aquaculture industry in Western Australia. As a result, there has been a long-term investment in surveys of commercial mollusc species for potential diseases of concern. A number of pathogens, particularly haplosporidians, identified within wild-stock shellfish have the potential to adversely affect mollusc populations. Others pose risks for translocations associated with aquaculture. The microsporidian *Stenhausia mytilovum* (Field), found in ova of the blue mussel *Mytilus galloprovincialis* (Lamarck), poses intriguing questions about the origin and dispersal of its host.

**KEY WORDS:** abalone, ciliates, parasites, *Perkinsus*, Thraustochytridea, scallops

### INTRODUCTION

The most valuable of the marine mollusc culture industries in Western Australia is pearling. The main species cultured is the golden-lipped pearl oyster *Pinctada maxima* (Jameson), but other pearl shells grown commercially include *P. margaritifera* (L.), *P. albina* (Lamarck) and *Pteria penguin* (Röding). The pearling industry is supported by four operational hatcheries capable of supplying all of the spat requirements of industry. Western Australia also has a small mussel industry based on culture of *Mytilus galloprovincialis*; an abalone culture industry based on hatchery produced *Halotis* spp. (greenlip abalone, *H. laevigata* (Donovan), brownlip abalone, *H. conicopora* Peron and Roe's abalone, *H. roei* Gray); a rock oyster farm hatching and growing *Saccostrea* sp.; a hatchery producing scallops (*Amusium balloti* Bernardi) for re-seeding and there is also some experimental re-seeding of tropical reefs with hatchery produced trochus (*Tectus niloticus* [L]).

Because of this aquaculture activity, the disease status of these Western Australian molluscs is relatively well known. To date there have been few diseases of concern. The health status of the aquaculture industry in a State such as Western Australia is based on two factors. The first is the presence of a disease reporting procedure and a diagnostic capacity to identify causative agents. The second is the existence of passive and targeted surveillance programs aiming to identify endemic diseases not associated with morbidity or mortality. In Western Australia a dedicated fish disease diagnostic unit of the Department of Fisheries has worked with industry since 1988 to identify and solve disease problems. There have also been 3 major mollusc disease surveys in the State; one involved a wide range of molluscs around the coast (Hine & Thorne 2000), one on *P. maxima* (Humphrey et al. 1998) and an ongoing national survey of abalone diseases. Some additional information is available in isolated publications on individual parasites and further unpublished information is available from laboratory records. Parasites that are known from shellfish in Western Australia are listed in Table 1.

### Pearl Oysters

The disease that has caused most economic loss to the *P. maxima* industry is vibriosis (Pass et al. 1987). In the 1980s *Vibrio* spp. bacteria caused significant losses to the pearling industry that

have now been overcome by improved management practices. The presence of a very rare haplosporidan in the digestive tubules is cause for concern (Hine & Thorne 1998) because very little is known about the biology of this parasite. The haplosporidan has been identified only three times and each time the oysters on the infected farm site have been destroyed. During the second occurrence, in late December 1995, 4.6% of a sample of 150 farmed juvenile pearl oysters was found infected within 6 weeks of being set at a remote site in Western Australia. By the time the oysters were destroyed 15 days later the infection prevalence had increased to 10% ( $n = 238$ ). The notifiable disease *Perkinsus olseni*/atlanticus has been reported from *P. maxima* in the Torres Strait (Norton et al. 1993a) but not from Western Australia, though the disease agent does occur here in bivalve molluscs other than *P. maxima* (Table 1).

Another parasite of concern in pearl oysters has only been detected in spat from the Exmouth Gulf and outlying islands since 2001, despite comprehensive monitoring of oysters in the State and the industry practice of harvesting and translocating shell to all other oyster growing areas for over 50 y. It is an intracellular ciliate similar in appearance to ciliates reported as nonpathogenic commensals in Spanish *Mytilus galloprovincialis* (Villalba et al. 1997) and in *Mytilus edulis* (L.) on the east and west coasts of North America (Figueras et al. 1991a). The teardrop shaped basophilic ciliate (10–15  $\mu\text{m} \times 5 \mu\text{m}$ ) has a dense polymorphic macronucleus and normally occupies an intraluminal or intraepithelial location within the digestive gland in *P. maxima* spat (Fig. 1). The ciliate is often associated with an inflammatory response in oysters smaller than 70 mm with 20–50 mm shell being most affected. A feature in smaller (20–40 mm) spat is the ciliate's capacity to penetrate the mucosal basal lamina and reside within hemolymph spaces or free within interstitial connective tissue. Translocation of infected pearl oysters beyond the Exmouth zone is not permitted.

The origin of the ciliate is unknown, and its relationship to similar organisms seen in northern hemisphere mussels is unknown. Either the ciliate has become much more prevalent in recent years or it has been introduced into the region. Its infectivity to other bivalves is also unknown.

During surveys to determine the distribution of the ciliate, a single infected oyster was found with an enigmatic unidentified proctistan parasite (Fig. 2). The putative sporoblasts develop within the epithelial cells of the digestive tubules and fill the lumen of the tubules, with an associated basophilic hemocyte inflamma-

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TABLE 1.  
Molluscan disease-causing agents (other than bacteria) reported from Western Australia.

Agent	Host	Associated with Epizootic?	Reference
<b>Virus</b>			
Virus-like inclusions	<i>Pinctada maxima</i>	No	Pass et al. (1988); Humphrey et al. (1998)
	<i>Haliotis laevis</i>	No	Laboratory records
	<i>Isognomon isognomonum</i>	No	Hine & Thorne (2000)
	<i>Pinctada bicolor</i>	No	Hine & Thorne (2000)
	<i>Saccostrea cucullata</i>	No	Hine & Thorne (2000)
	<i>Haliotis laevis</i>	No	Laboratory records
Papova-like virus	<i>Pinctada maxima</i>	No	Norton et al. (1993b); Humphrey et al. (1998)
<b>Rickettsia-like organisms</b>			
	<i>Barbatia helblingii</i>	No	Hine & Thorne (2000)
	<i>Haliotis laevis</i>	No	Laboratory records
	<i>Isognomon isognomonum</i>	No	Hine & Thorne (2000)
	<i>Pinctada maxima</i>	No	Humphrey et al. (1998)
	<i>Pinctada bicolor</i>	No	Hine & Thorne (2000)
	<i>Pinctada deltoidea</i>	No	Hine & Thorne (2000)
	<i>Pteria penguin</i>	No	Hine & Thorne (2000)
	<i>Saccostrea glomerata</i>	No	Hine & Thorne (2000)
	<i>Saccostrea cucullata</i>	No	Hine & Thorne (2000)
	<i>Stavilia horrida</i>	No	Hine & Thorne (2000)
<b>Haplosporidia</b>			
<i>Bonamia</i> sp.	<i>Ostrea</i> sp.	Yes	Laboratory records
<i>Haplosporidium</i> sp.	<i>Saccostrea cucullata</i>	Yes	Hine & Thorne (2000)
<i>Haplosporidium</i> sp.	<i>Pinctada maxima</i>	No	Hine & Thorne (1998)
<i>Marteilia sydneyi</i>	<i>Saccostrea cucullata</i>	No	Hine & Thorne (2000)
	<i>Saccostrea glomerata</i>	No	Hine & Thorne (2000)
<i>Mikrocytos roughleyi</i>	<i>Saccostrea</i> sp.	No	Laboratory records
<b>Microspora</b>			
<i>Steinhausia mytilovum</i>	<i>Mytilus galloprovincialis</i>	No	Laboratory records
<i>Marteilioides</i> sp.	<i>Saccostrea echinata</i>	No	Hine & Thorne (2000)
<b>Apicomplexa</b>			
Heart apicomplexan	<i>Pinctada maxima</i>	No	Humphrey et al. (1998)
<i>Perkinsus olseni</i>	<i>Barbatia helblingii</i>	No	Hine & Thorne (2000)
	<i>Isognomon isognomonum</i>	No	Hine & Thorne (2000)
	<i>Malleus meridianus</i>	No	Hine & Thorne (2000)
	<i>Pinctada albina</i>	No	Hine & Thorne (2000)
	<i>Pinctada deltoidea</i>	No	Hine & Thorne (2000)
	<i>Saccostrea cucullata</i>	No	Hine & Thorne (2000)
	<i>Saccostrea glomerata</i>	No	Hine & Thorne (2000)
	<i>Septifer bilocularis</i>	No	Hine & Thorne (2000)
	<i>Spondylus</i> sp.	No	Hine & Thorne (2000)
	<i>Haliotis laevis</i>	No	Laboratory records
<b>Ciliates</b>			
Ciliates on gills	<i>Pinctada</i> spp.	No	Humphrey et al. (1998)
	<i>Saccostrea</i> sp.	No	Laboratory records
Ciliates in gut	<i>Pinctada maxima</i>	No	Laboratory records
	<i>Haliotis laevis</i>	No	Laboratory records
Ancistrocomids	<i>Pinctada albina</i>	No	Hine & Thorne (2000)
	<i>Pinctada bicolor</i>	No	Hine & Thorne (2000)
	<i>Saccostrea cucullata</i>	No	Hine & Thorne (2000)
	<i>Saccostrea echinata</i>	No	Hine & Thorne (2000)
	<i>Saccostrea glomerata</i>	No	Hine & Thorne (2000)
	<i>Haliotis laevis</i>	No	Laboratory records
<i>Sphenophyra</i> -like ciliates	<i>Saccostrea glomerata</i>	No	Hine & Thorne (2000)
<b>Trematoda</b>			
<i>Proctoeces</i> sp.	<i>Malleus meridianus</i>	No	Hine & Thorne (2000)
Sporocysts	<i>Haliotis laevis</i>	No	Laboratory records
	<i>Isognomon isognomonum</i>	No	Hine & Thorne (2000)
	<i>Pinctada albina</i>	No	Hine & Thorne (2000)
	<i>Pinctada maxima</i>	No	Humphrey et al. (1998); Pass (1987); Hine & Thorne (2000)
	<i>Malleus malleus</i>	No	Hine & Thorne (2000)
	<i>Saccostrea echinata</i>	No	Hine & Thorne (2000)
	<i>Saccostrea glomerata</i>	No	Hine & Thorne (2000)

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TABLE 1.  
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Agent	Host	Associated with Epizootic?	Reference
<b>Cestoda</b>			
<i>Fylocephalum</i> sp.	<i>Dendostrea folium</i>	No	Hine & Thorne (2000)
	<i>Isognomon isognomum</i>	No	Hine & Thorne (2000)
	<i>Malleus malleus</i>	No	Hine & Thorne (2000)
	<i>Malleus meridianus</i>	No	Hine & Thorne (2000)
	<i>Pinctada</i> spp.	No	Hine & Thorne (2000); Humphrey et al. (1998)
	<i>Pinna bicolor</i>	No	Hine & Thorne (2000)
	<i>Pinna deltoidea</i>	No	Hine & Thorne (2000)
	<i>Pteria penguin</i>	No	Hine & Thorne (2000)
	<i>Saccostrea cucullata</i>	No	Hine & Thorne (2000)
	<i>Saccostrea glomerata</i>	No	Hine & Thorne (2000)
	<i>Saccostrea echinata</i>	No	Hine & Thorne (2000)
	<i>Stavilia horrida</i>	No	Hine & Thorne (2000)
Tetrabothriate cestodes	<i>Pteria penguin</i>	No	Hine & Thorne (2000)
<b>Nematoda</b>			
Nematode larvae	<i>Saccostrea cucullata</i>	No	Hine & Thorne (2000)
<i>Sulcascaris sulcata</i>	<i>Amisium balloti</i>	No	Lester et al. (1980)
<i>Echinocephalus</i> sp.	<i>Amisium balloti</i>	No	Lester et al. (1980)
<b>Gregarines</b>			
<i>Nematopsis</i> sp.	<i>Saccostrea cucullata</i>	No	Hine & Thorne (2000)
Unidentified gregarine	<i>Pinctada maxima</i>	No	Humphrey et al. (1998)
<b>Copepoda</b>			
<i>Anthessius pinctadae</i>	<i>Pinctada maxima</i>	No	Humphrey et al. (1998)

tory response. With a prevalence of less than 0.005% it is not feasible to attempt follow-up sampling, but it does indicate that parasitic organisms can be present at extremely low prevalence in a population.

Another proctistan was detected at a low prevalence in *P. maxima* from the Exmouth Gulf during investigations into the ciliate parasite. The parasite was elongated (30  $\mu\text{m} \times 20 \mu\text{m}$ ) and intimately associated with the digestive gland epithelial cells to which it appeared to have a sessile attachment (Fig. 3). The tubule epithelial cells beneath the site of attachment were multinucleated and ultrastructural examination indicated the multiple nuclei were of molluscan origin, suggesting the proctistan had induced this change within the host.

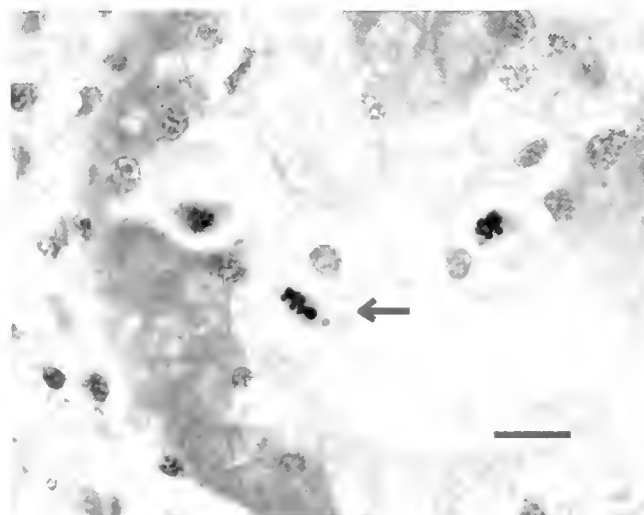


Figure 1. Ciliate in digestive tubules of *Pinctada maxima* spat from Exmouth Gulf (arrow). H&E, scale bar = 10  $\mu\text{m}$ .

A proctistan tentatively attributed to the Thraustochytridea was identified in moribund, gaping *P. maxima* from a farm that had experienced losses after a cyanobacterial (*Trichodesmium* sp.) bloom. The affected oysters showed extensive necrosis of external epithelial surfaces of the palps and mantle with invasion of the underlying leydig tissues by brown pigmented and eosinophilic, segmented unicellular organisms 10–15  $\mu\text{m}$  in diameter, and smaller dense basophilic 5- $\mu\text{m}$  diameter cells that appeared to be embedded in a mucinous matrix (Fig. 4).

#### Rock Oysters

One of 411 *Saccostrea glomerata* (Gould) collected between Carnarvon to the Dampier Archipelago during 1995 was infected

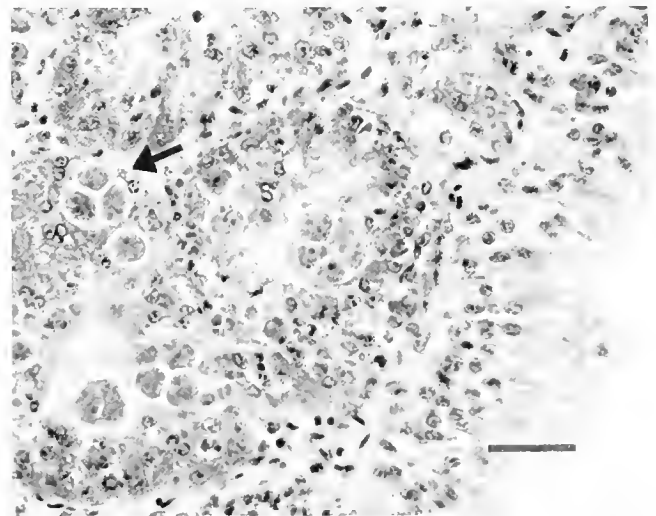


Figure 2. Unidentified proctistan parasite (arrow) in pearl oysters *Pinctada maxima* from Exmouth Gulf. The parasite fills the digestive gland. H&E, scale bar = 30  $\mu\text{m}$ .

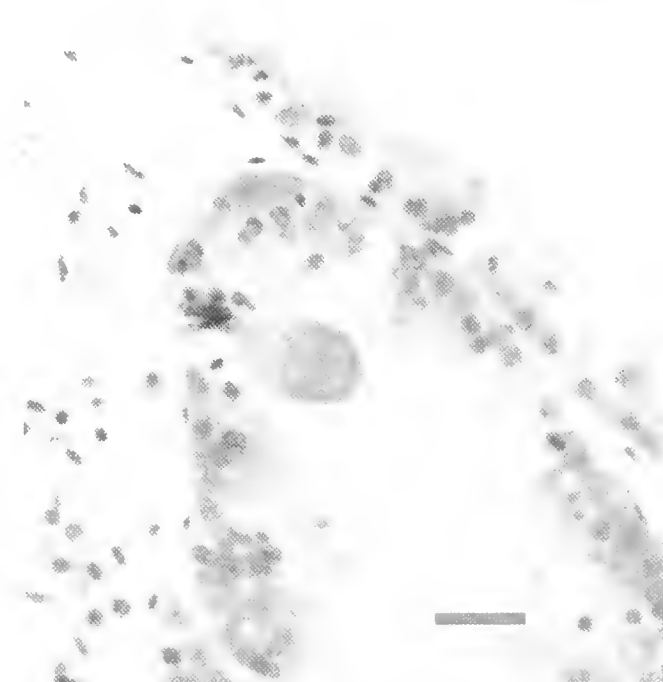


Figure 3. A large proctistan intimately attached to digestive tubule epithelial cells by apparent cytoplasmic attachments with hyperplasia of tubule nuclei, in *Pinctada maxima*. H&E, scale bar = 10 µm.

with a parasite histologically identical to *Marteilia sydneyi* Perkins and Wolf, the cause of QX disease in *Saccostera commercialis* (Fredale and Roughley) in Queensland and New South Wales. The same pathogen was also found in *S. glomerata* by Hine and Thorne (2000). On the eastern seaboard where QX occurs, it has caused losses of over 90% (Witney et al. 1988) and has resulted in a decline in New South Wales rock oyster production since the 1970s.

*S. cucullata* (Born) from Airlie Island, Rosily Island, Varanus Island, Hermite Island, King Bay and East Lewis Island in the

north of Western Australia are infected by a second species of pathogenic haplosporidan protozoan, *Haplosporidium* sp. The parasite was identified as the cause of a significant mortality (up to 80%) of rock oysters at Airlie Island (Hine & Thorne 2000). The presence of this haplosporidan will threaten the viability of any rock oyster farm sited north of Exmouth.

#### Mussels

The ova of mussels in Cockburn Sound, Western Australia are infected with the microsporidian *Steinhausia mytilovum* but the parasite has not been found in mussels from other growing areas. Heavily infected mussels are readily identified by the gross appearance of the mantle of cooked females. Infected mantle tissue has an uneven surface with depressed cream white patches and swollen tubercles forming spots against the orange-pink background color of healthy female gonad. Mussel samples in August and October 1995 from sites around the Sound showed a prevalence of *Steinhausia* sp. ranging from 22% to 57%, or 44.4% overall. Histology revealed that the patches and tubercles were associated with a marked infiltration of circulating granulocytes and large basophilic hemocytes into affected follicles with resorption of the germinal epithelium and ova (Fig. 5). Small basophilic hemocytes were not prominent in the response. Also present in 2% to 4% of the ova were parasitophorous vacuoles closely associated with the nucleus. These vacuoles measured  $13.27 \pm 2.37$  µm ( $n = 20$ ) and contained over 30 small spores 2.5-µm diameter. There was usually only one vacuole in an ovum, but occasionally up to three parasitophorous vacuoles could be seen. In histological sections the presence of resorbing follicles and focal accumulation of granulocytes among the developed ova was evidence of infection, but infected oocytes were also observed in apparently healthy follicles. Ova (normal, infected and degenerating), granulocytes, large basophilic granular hemocytes and cell debris occurred in the ciliated gonad ducts. Parasitophorous cysts containing spores also occurred free in the lumen of the ducts. The spore walls and contents stained negative to Feulgen, Ziehl-Neelsen and Grocotts, were Gram positive and Periodic-Acid-Schiffs negative.

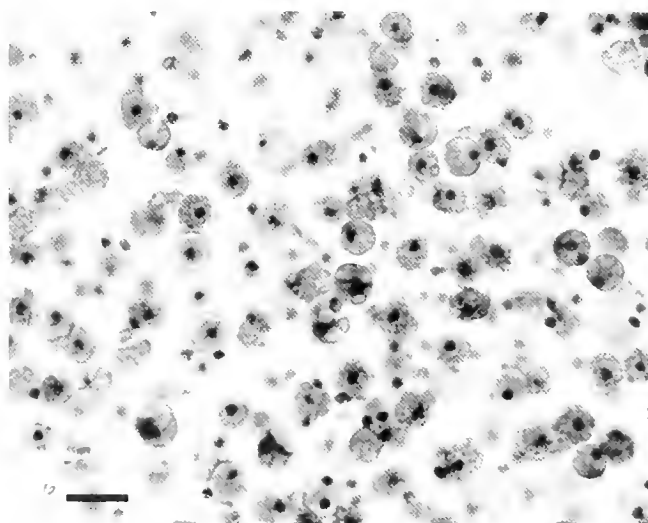


Figure 4. Large 15 µm dia. segmented refractile eosinophilic cells and smaller 5-µm basophilic cells within the leydig tissues of *Pinctada maxima*, tentatively attributed to a thransstochytrid. H&E, scale bar = 20 µm.

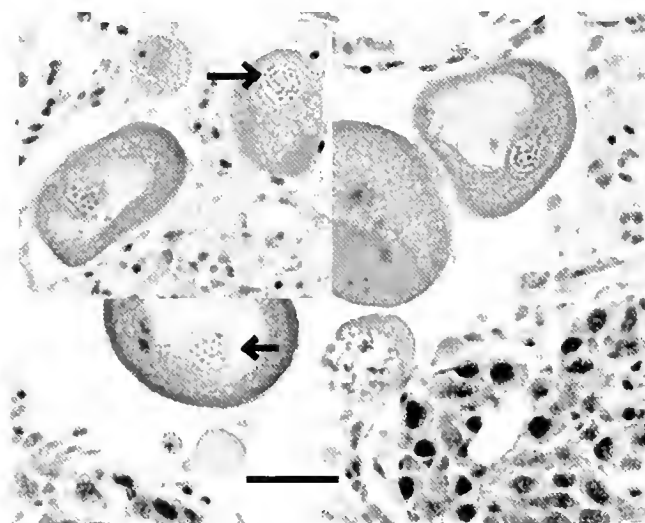


Figure 5. *Steinhausia mytilovum* in *Mytilus galloprovincialis* from Cockburn Sound, Western Australia (arrows), main picture and insert. Note inflammatory response in main picture. H&E, scale bar = 10 µm.

The relationship between this *Steinhausia* sp. and the one previously described by Anderson et al. (1995) from cysts within ova of the rock oyster *S. commercialis* in Queensland, Australia is unknown. The biology of *Steinhausia* sp. is not well understood. Field (1923) reported that infected eggs were shed along with normal eggs. Sparks (1985) suggested that infected eggs do not seem to become necrotic or degenerate though Rybakov and Kholodkovskaya (1987) noted that *Steinhausia* sp. clearly distorts the nucleus of the ovum and can also cause the destruction of the egg, as has been seen in Western Australian mussels. It is likely that the loose spores are released along with intact eggs or through phagocytosis and subsequent diapediasis. Figueras (1991) reported that the presence of the parasite is always accompanied by a strong hemocyte response and the impact on the host has been described as severe (Rybakov & Kholodkovskaya 1987) to negligible (Maurand & Loubès 1979). In the case of the *S. mytilorum* infection seen here, there is an absence of the typical bivalve inflammatory response, which involves invasion of the site of trauma primarily by small agranular hyalineocytes (90%), granular basophils (8%) and granular acidophils (2%) (Bayne et al. 1979, Brereton & Alderman 1979). Instead the major components are the phagocytic hemocyte and the granular acidophil and the process closely resembles gonad resorption. Prevalence of *Steinhausia mytilorum* does not increase with the size of the mussel, suggesting that infection is annual (Table 2).

The proctistan observed in the ova of Western Australian blue mussel has the same measurements and appearance under the light microscope as *S. mytilorum* from both European and American *M. galloprovincialis*. The taxonomy of the blue mussel in Western Australia is disputed. Still referred to as *M. edulis planulatus* (L.), electrophoretic studies have shown that the species is *M. galloprovincialis* and that *M. galloprovincialis* from Australasia, eastern Asia, Western Europe, the Mediterranean and California, and *M. edulis* from eastern North America and Western Europe are electrophoretically distinct species with an overlapping distribution (Koehn 1991, McDonald et al. 1991, Geller et al. 1993). There is a fossil record of mussels in Australian waters and Koehn (1991) hypothesized that *M. galloprovincialis* may have been an early introduction into the Northern Hemisphere as a hull-fouling organism. Distribution of parasites often reflects the distribution of their primary hosts so *S. mytilorum* may also be an introduction from the Southern Hemisphere. *Steinhausia mytilorum* is reported to infect the ova of *M. edulis* along the Atlantic coast of the USA (Field 1923, Figueras et al. 1991a). Sparks (1985) reported that,

based on examination of thousands of mussels, the parasite was absent from California, Oregon and Washington and it was unreported from Europe. It now occurs in *M. galloprovincialis* from Spain (Figueras et al. 1991b), Italy (De Vincentiis & Renzoni 1963), Greece (Rayyan et al. 2004), the Black Sea (Rybakov & Kholodkovskaya 1987, Gayevskaya & Machkevskiy 1991), northern France (Comtet et al. 2004) and the west coast of the USA (Hillman 1990, 1991). Hillman (1990) noted that *M. galloprovincialis* had been accidentally introduced into southern California and suggested that *S. mytilorum* had been introduced with the mussels.

#### Scallops

There are two nematode larvae found in scallops (*Amusium balloti*) in the Shark Bay area of Western Australia. The "common" nematode in scallops is *Sulcascaris sulcata*. This was reported by Lester et al. (1980) to infect up to 64% of the landed catch in Shark Bay and occurs in a brownish capsule 3–7 mm dia. The adult nematodes of *S. sulcata* live in the loggerhead turtle *Caretta caretta* (L) and have a wide geographic distribution and range of molluscan hosts. The second species, *Echinocephalus* sp., forms small yellow-brown cysts 2–3 mm dia. Lester et al. (1980) reported that only 2 of 10 scallops he examined were infected, but in recent years the nematode has been much more common and in 2001 was the dominant nematode in *A. balloti*. The genus *Echinocephalus* occurs widely in molluscs in warm waters, and probably matures in marine skates or rays.

#### Abalone

The Western Australian component of a national health survey of abalone has recently been completed. In this survey, up to 25% of wild-caught green-lipped abalone (*Haliotis laevis*) were infected with trematode metacercariae. Low prevalences of proctistans in the lumen of the stomach and digestive gland, apicomplexans and putative viral inclusions in the intestinal tract were observed. Abalone in Western Australia are free of the disease perkinsosis, found in South Australia and New South Wales, but the organism does exist on the south coast of Western Australia. *Perkinsus* is a primitive fungus-like organism of uncertain taxonomic status, probably in the phylum Labrithulomycota. A worldwide effort to understand the taxonomy of this organism (or group of organisms) is underway. In South Australia and New South Wales *Perkinsus olseni/atlantensis* affects abalone with yellow-green pus filled blisters (0.5–8 mm dia.) containing a creamy brown deposit. Once processed the lesions appear as pale brown circles. Perkinsosis occurs in a variety of shellfish in the north of the State (Hine & Thorne 2000), however, a survey of 300 abalone from six sites along the south and west coasts of Western Australia in 1995 were negative for *Perkinsus* sp. by the thioglycolate media method. Subsequently, in 2003 *Perkinsus* sp. was cultured from the gill tissue of one clinically normal abalone (*H. laevis*) from the south coast.

#### FUTURE TRENDS

Whereas, overall, disease has not been a problem for the mollusc industry in Western Australia, it is certain that many more pathogenic organisms remain to be discovered, particularly as molluscs become subject to aquaculture or are subject to environmental stresses associated with economic activity. Because of the age

TABLE 2.

Prevalence of *Steinhausia* sp. in female *Mytilus galloprovincialis* from Cockburn Sound, Western Australia.

Size of host (mm)	Number infected	Total number examined	% infected
<50	16	34	47.0
50–59	33	65	50.7
60–69	23	80	28.7
70–79	93	189	49.2
80–89	51	114	44.7
>90	7	10	70.0
Overall	223	492	45.3

There is no increase in prevalence of infection with mussel size.

of the Australian continent and the relative isolation of the coastal fauna it is likely that many of these will prove to be unique to Western Australia. Strict controls are therefore imposed to limit translocation of parasites by aquaculture. Whereas these pathogens may represent an economic threat, it is probable that they will also provide new insights on the zoogeography and derivation of the Western Australian mollusc fauna.

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## GROWTH AND SURVIVAL OF JUVENILE GREENLIP ABALONE (*HALIOTIS LAEVIGATA*) FEEDING ON GERMLINGS OF THE MACROALGAE *ULVA* SP

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**ABSTRACT** Germlings of the green alga *Ulva* sp. were developed as a diet for juvenile *Haliotis laevigata* ( $\geq 3.5$  mm shell length) and compared with a current commercial diet consisting of *Ulva lens* plus the diatom species *Navicula cf. jeffreysi*. The utilization of macroalgae germlings (juvenile gametophyte and sporophyte) allowed 3-dimensional growth and subsequently provided greater feed biomass in comparison with the current 2-dimensional commercial feed for the later nursery phase consisting of 5–10 mm (shell length) juvenile abalone. The juvenile abalone showed active feeding on both the *Ulva* germling diet and the current commercial diet. The *Ulva lens*/*Navicula cf. jeffreysi* diet resulted in abalone of significantly larger shell length at the end of the 14-wk feeding trial. However, the *Ulva* germling diet recorded significantly larger abalone for the first 4–5 wk, whereas the commercial diet produced significantly larger abalone from week 6 to the end of the trial. The growth rate on both diets exceeded  $100 \mu\text{m}\cdot\text{day}^{-1}$  and the specific growth rates were maintained above  $1\%\cdot\text{day}^{-1}$  for the duration of the feeding trial with neither measure portraying significant differences between diets. There was no significant difference in juvenile abalone mortality feeding on the two diets. The *Ulva* germling consumption exhibited a spike ( $500 \text{ germling blades}\cdot\text{abalone}^{-1}\cdot\text{day}^{-1}$ ) in consumption at week three then, once reduced, a gradual increase occurred until the end of the trial. *Ulva lens* consumption demonstrated a similar pattern to *Ulva* germlings consumption and was significantly, positively correlated. Consumption rates for the two green algae both correlated with juvenile abalone growth. The diatom (*Navicula cf. jeffreysi*) consumption was affected by plate rotation (light intensity and grazing pressure) rather than juvenile abalone.

**KEY WORDS:** juvenile abalone, *Haliotis laevigata*, *Ulva*, germlings, *Ulva lens*, diatoms, dietary value

### INTRODUCTION

To culture quality abalone to commercial harvest size within an economical time frame, current culture protocols and in particular, juvenile nutrition need to be improved. To advance this area of production, new juvenile diets must be explored that supply sufficient biomass and provide greater nutritional benefits.

The main diet of postlarval and early juvenile abalone (up to ~5 mm) in the natural environment consists of epiphytic and epilithic diatoms, crustose coralline algae, turf algae and bacteria, whereas larger juveniles consume macroalgae (Dunstan et al. 2002, Kawamura et al. 1995, Kawamura 1996, Kawamura & Takami 1995, McShane et al. 1994, Takami et al. 1998). Once abalone reach the transition phase from a diatom-based diet to a macroalgae diet, diatoms such as *Cylindrotheca closterium* (Ehrenberg) alone are no longer sufficient to maintain adequate growth rates in an aquaculture system (Takami et al. 2003). At this stage additional algal food is required to sustain maximum growth rates and reduce the variability of growth and survival rates. Maintenance of an adequate food supply to the 5–10 mm juveniles is seen as a major limiting factor in the intensification of abalone nurseries (Krsinich et al. 2000).

Currently in Australian commercial abalone nurseries, postlarvae are supplied with diatoms (e.g., *Navicula cf. jeffreysi*) and as they develop into juveniles they are provided with the crustose green alga *Ulva lens* crouch (Daume & Ryan 2004, Daume et al. 2004). *U. lens* has been shown to induce higher settlement rates of abalone larvae compared with monospecific benthic diatom films (Daume et al. 2000, Krsinich et al. 2000). By itself *U. lens* only supports moderate growth rates but, when combined with an easily digestible diatom such as *N. jeffreysi*, the diet can sustain high

growth rates (Daume & Ryan 2004, Kawamura et al. 1998). Takami et al. (1997) also found that rapid abalone growth is only achievable on crustose coralline algae (*Lithophyllum yes-soense*) if diatoms are present. Once abalone exceed about 5 mm in length, the combined diet of *U. lens* and *N. jeffreysi* is unable to adequately support the high abalone biomass per plate (Daume & Ryan 2004).

A potential alternative commercial feed for juvenile abalone (5–10 mm) may be macroalgae sporelings. The majority of juvenile abalone dietary studies have been conducted with mature macroalgae; however, they may have different nutritional and structural properties to juvenile macroalgae of the same species (Van Alstyne et al. 1999). The juvenile macroalgae (germlings) can potentially provide a greater biomass per plate because of their 3-dimensional morphology compared with the 2-dimensional encrusting algae and have been shown to support moderate to rapid growth of  $90\text{--}130 \mu\text{m}\cdot\text{day}^{-1}$  (Maesako et al. (1984) as cited in Kawamura et al. (1998)).

The 3-dimensional growth reduces the surface area required and gives the feed the potential to combat the juvenile abalone's ability to consume 5% to 30% of their body weight in algae each day (Corazani & Illanes 1998, Hahn 1989). *Ulva* has been used in numerous studies, both individually or as part of mixed/rotation diets but is considered a relatively poor nutrition source (Simpson & Cook 1998). Shpigel et al. (1999) has shown that specific growth rates of  $0.6$  to  $1\%\cdot\text{day}^{-1}$  can be attained for juveniles 8–15 mm in shell length and that some abalone species grow better on *Ulva* cultured in high ammonia-N enriched seawater, underlying the importance of the feed's nutritional value.

In this study, the dietary value of an *Ulva* sp. germling diet was compared with a currently used commercial diet consisting of the green alga *U. lens* plus the diatom species *N. jeffreysi* on the growth and survival of juvenile greenlip abalone (*Haliotis laevigata* Donovan).

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## MATERIALS AND METHODS

### Location

The feeding trial was conducted in a greenhouse at the Aquaculture Development Unit, Challenger TAFE, Fremantle, Western Australia between March and August 2004. Juvenile greenlip abalone (*Haliotis laevis*) were supplied by Great Southern Marine Hatcheries in Albany, Western Australia.

### Algal Culture—Diets

#### *Ulva* sp. Germling Diet

*Ulva* sp. thalli were collected from submerged limestone rocks on South Mole in Fremantle and exposed to a cold (4 °C) treatment to induce gametogenesis. *Ulva* thalli were arranged in layers in-between moist newspaper then refrigerated. After 7 days of cold treatment, 10 kg blotted wet weight of *Ulva* thalli was placed into each of the five, 400 L tanks filled with a modified f/2 culture medium (Guillard & Ryther 1962), that lacked PII metals, sodium metasilicate and vitamin stock solutions. Each tank held 3 baskets of 12, 30 × 60 cm PVC plates lying horizontally. The tanks received only light aeration to reduce water motion and allow maximum spore attachment.

The *Ulva* thalli were removed from the five tanks after 6 days and the germling seeded PVC plates redistributed into three 400 L tanks each containing 3 baskets of 20 plates now orientated vertically. The germlings were then cultured over 5 wk in the modified f/2 medium, which was exchanged twice weekly.

#### *Ulvella lens* Plus *Navicula cf. jeffreysi* Diet

The diatom *Navicula cf. jeffreysi* (CSIRO Hobart, CS-514) was cultured in standard f/2 medium (Guillard & Ryther 1962) with cultures starting indoors in 4 petri dishes that were then scaled up through four, 1.5 L horizontally laid culture bags and finally to one, 60 L, shallow tank outdoors. 20 L of the *N. jeffreysi* inoculum was added to each of the three *U. lens* tanks.

Sixty *U. lens* seed plates (30 × 60 cm PVC) (Daume & Ryan 2004, Daume et al. 2004) were placed at regular intervals between clean 30 × 60 cm PVC plates and exposed to sunlight for 5 days, then removed. The aeration was low to allow the *U. lens* spores to attach to the plates and the modified f/2 medium was exchanged twice weekly.

### Feeding Trial

For each of the 2 treatments (*Ulva* germling diet and *U. lens/N. jeffreysi* diet), three, 400 L tanks were stocked with three baskets of 20 vertically arranged seeded plates (30 × 60 cm). The tanks were aerated by three airlines spaced evenly along the bottom and shaded with 70% shade cloth; 1 µm filtered bore seawater was supplied at 10 L.min<sup>-1</sup> via a spray bar above the water surface. The water temperature over the 3 mo feeding trial started at 20.8 ± 0.13 °C (May) then reduced to 19.7 ± 0.18 °C (June) and finished at 19.0 ± 0.08 °C (July).

Juvenile greenlip abalone (*H. laevis*) were taken off an *U. lens*/naturally occurring diatoms diet and transported (4 h) on PVC plates seeded with *U. lens* between wet sponge sheets in insulated containers. The PVC plates with juveniles attached were placed across the top of the baskets in each tank and left for 2 wk to allow the juveniles to migrate onto the experimental diet plates. Seventeen hundred juveniles of 3.5–4 mm shell length were stocked in

each of the six tanks giving approximately 28 juveniles per 30 × 60 cm plate.

The feeding trial was for a period of 14 wk and the three *U. lens* tanks were reinoculated with *N. jeffreysi* during weeks 2, 4 and 8. The plates in all tanks were rotated twice, 180° about the horizontal in weeks 3 and 10.

### Measurements

Abalone shell length (mm) and weight (g) were measured at the beginning of the trial and then weekly by collecting a sub sample of 50 juveniles from 10 randomly selected plates in each tank. After the juvenile abalone had been measured the contents of each tank were siphoned through 50 µm mesh and the dead abalone counted.

*Ulva* germling abundance was determined by counting the number of germling blades per cm<sup>2</sup> of plate at weekly intervals. Every fifth plate was counted with 5 randomly selected fields of view (0.785 cm<sup>2</sup>) counted diagonally across the plate. The density of the *U. lens* was determined by estimating percentage cover along a graticule using the same sampling procedure as the *Ulva* germlings. The density of *N. jeffreysi* was measured on 2 removable notches cut from the side of every sixth plate. The notches were approximately 16 cm<sup>2</sup> and positioned 3 cm from the top and bottom. The number of cells present on these notches was then counted for a defined area in 20 randomly chosen fields of view and the number of cells.cm<sup>-2</sup> calculated.

### Biochemical Analysis

Samples were taken by scraping diagonally across the plates that were used for determining weekly algal abundance. Scrapings were stored at -20 °C until needed.

### Algal Dry Weight

Five milliliters of the *U. lens/N. jeffreysi* samples and 0.05 g of the *Ulva* germling sample were filtered through Whatman GF/C (2.5 cm) glass microfiber filters that had been washed, precombusted and preweighed. The filtrate was then washed with 10 mL of ammonium formate solution (0.65 M) to remove excess salts, dried in an oven for 12 h (80 °C) and placed in a vacuum desiccator overnight. They were then weighted to 4 decimal places on an analytical balance.

### Lipid Determination

The lipid content of the algal diets were determined based on the method of Bligh and Dyer (1959) as modified by Kates and Volcani (1966) and adapted by Mercz (1994).

Five milliliters of the *U. lens/N. jeffreysi* samples and 0.025 g of the *Ulva* germling samples were filtered onto Whatman GF/C (2.5 cm) glass microfiber filters, rinsed with 10 mL ammonium formate (0.65 M) and stored at -20 °C for approximately 2 mo.

Once thawed, filters were homogenized in a glass mortar and pestle with 5 mL of a methanol:chloroform:deionised water solution (2:1:0.8 v/v/v). The extract was centrifuged at 3000 rpm for 5 min and the supernatant transferred to a second, 10 mL graduated glass centrifuged tube. The volume was made up to 5.7 mL with fresh methanol:chloroform:deionised water, then 1.5 mL chloroform and 1.5 mL deionized water were added while mixing well.

The tubes were recentrifuged (3,000 rpm for 5 min), after which phase separation was complete and the lower green chloroform layer containing the lipids were carefully transferred into



dry, preweighted 4 mL glass vials. A few drops of toluene were added and the extract dried under ultra pure nitrogen. The vials were placed in a vacuum desiccator (KOH pellets) overnight and weighted to 4 decimal places.

#### Protein Determination

The protein content of the algal diets were determined utilizing a modification of the Lowry et al. (1951) method by Dorsey et al. (1978) and Mercz (1994). Samples were prepared as in the Lipid Determination procedure (above) with 0.0125 g of *Ulva* germlings used for each sample.

Filters were homogenized with 5 mL Biuret reagent in a glass mortar and pestle, then transferred into 10 mL graduated glass centrifuged tubes and 0.14 mL of deionized water added. Protein standards (Bovine Serum Albumin) of 0, 10, 20, 30, 40, 50, 60 and 70  $\mu\text{g}$  were made up to 0.14 mL with deionized water, and 5 mL Biuret reagent was added.

All tubes were incubated at 100°C for 60 min and immediately after 0.5 mL Folin Phenol reagent was added while mixing on a Vortex stirrer. The tubes were cooled for 15 min at 10°C to 15°C and 15 min at room temperature, then centrifuged (3,000 rpm for 5 min). The absorbance of the supernatant was read at 660 nm and the protein content determined from the standard curve.

#### Carbohydrate Determination

The carbohydrate content of the algal diets were determined using the method of Kochert (1978) incorporating modifications by Ben-Amotz et al. (1985) and Mercz (1994). Samples were prepared as in the lipid determination procedure (earlier) with 0.012 g of *Ulva* germlings being used.

Five milliliters of  $\text{H}_2\text{SO}_4$  (1 M) was used to homogenize filters in a glass mortar and pestle before being transferred into 10 mL graduated glass centrifuged tubes and incubated at 100°C for 60 min. After cooling to room temperature and centrifuging (3,000 rpm for 5 min) a known volume of supernatant (<50  $\mu\text{g}$  total carbohydrate, which is between 0.1–0.5 mL, depending on initial algal concentration) was taken and made up to 2 mL with deionized water in 10 mL graduated glass centrifuged tubes. Carbohydrate standards (Glucose) of 0, 10, 20, 30, 40 and 50  $\mu\text{g}$  were made up to 2 mL with deionized water.

One milliliter of 5% (w/v) phenol solution was added and mixed well on a Vortex stirrer. Five milliliter of concentrated  $\text{H}_2\text{SO}_4$  (98%, 18 M) was added rapidly and then the tubes left for 30 min to cool. Absorbance was measured at 485 nm and the carbohydrate content determined from the standards.

#### Data Analysis

Juvenile abalone growth and density for the 2 dietary treatments *Ulva* germling and *U. lens/N. jeffreyi* were compared by analysis of variance (1-way ANOVA). A univariate analysis of variance, posthoc (Tukey HSD) test was applied to test for differences between mean abalone interval sizes (shell length) on the two diet treatments at each time interval. Comparisons of the algae diets consumption and biochemical composition were achieved through Bivariate Correlation and analysis of variance (1-way ANOVA) respectively. The plate rotation was analyzed with an Independent *t*-test.

## RESULTS

#### Abalone Growth

At the commencement of the feeding trial there was no significant difference between the average shell lengths of the abalone

distributed to the 2 diets ( $F_{(df\ 1,298)} = 0.49$  [ $P = 0.484$ ]). The juvenile abalone grew on both diets with the *Ulva* germling diet producing significantly larger abalone (shell length) for the first 5 wk ( $F_{(df\ 1,2398)} = 6.779$  [ $P < 0.05$ ]) (Fig. 1).

During the first 4 wk the mean weekly increase in shell length of the abalone on the *Ulva* germling diet was  $0.51 \pm 0.1$  mm with each increase shown to be significant (Table 1). The abalone on the *U. lens/N. jeffreyi* diet only averaged a weekly increase in shell length of  $0.41 \pm 0.1$  mm for the first 4 wk but were able to maintain significant increases in shell length until week 7 (Table 1). The subsequent extended period of significantly faster growth resulted in the abalone on the *U. lens/N. jeffreyi* diet surpassing the size (shell length) of the abalone on the *Ulva* germling diet and this transition is evident in Figure 1 where the two growth profiles intersect between weeks 5 and 6. The *U. lens/N. jeffreyi* diet then proceeded to yield significantly larger abalone (shell length) ( $F_{(df\ 1,2698)} = 24.671$  [ $P < 0.05$ ]).

The weekly growth rates of juvenile abalone were very variable (reaching over  $100\ \mu\text{m}\cdot\text{day}^{-1}$ ) on both the *Ulva* germling diet and the *U. lens/N. jeffreyi* diet during the first 6 wk (Table 1, Table 2). The *Ulva* germling diet sustained a higher specific growth rate for abalone over the first 4 wk, reaching a maximum of  $1.5\%\cdot\text{day}^{-1}$  (Fig. 2).

After the 8th wk of the feeding trial, shell length was not significantly different between adjacent weeks on either diet (Table 1), indicating a reduction in absolute growth rate (Table 2) and specific growth rate (Fig. 2). From Table 2 it is evident that the *Ulva* germling diet produced slightly lower, not significantly lower, ( $F_{(df\ 1,82)} = 0.583$  [ $P = 0.448$ ]) growth rates over the entire feeding trial. Consequently, the specific growth rate of the juvenile abalone was not significantly affected by diet ( $F_{(df\ 1,82)} = 1.968$  [ $P = 0.164$ ]) with both recording  $1\%\cdot\text{day}^{-1}$  by the end of the 14 wk trial (Fig. 2).

The juveniles consuming the *Ulva* germling diet were smaller at the completion of the trial with an average of  $9.61 \pm 0.1$  mm shell length, compared with the average shell length of  $10.29 \pm 0.1$  mm the abalone on the *U. lens/N. jeffreyi* achieved (Fig. 1). The final abalone shell lengths were significantly different indicating that the *U. lens/N. jeffreyi* diet produced significantly larger abalone than the *Ulva* germling diet ( $F_{(df\ 1,298)} = 10.335$  [ $P < 0.05$ ]).

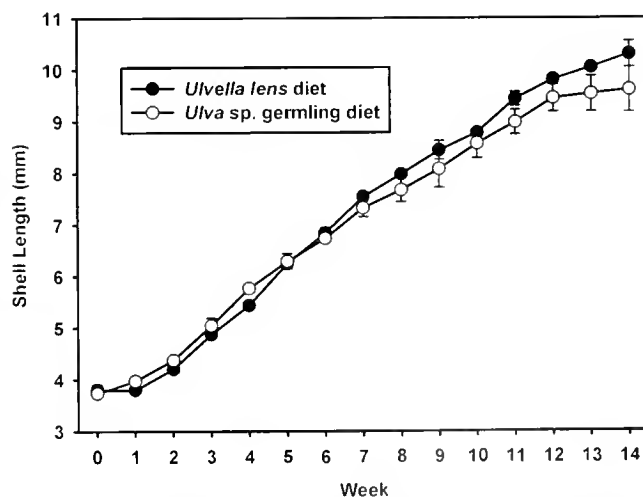


Figure 1. Growth (shell length) of juvenile *Haliotis laevis* over the 14 wk feeding trial on an *Ulva* germling diet and an *Ulva lens/Navicula cf. jeffreyi* diet. Mean  $\pm$  std. error ( $n = 3$ ).

TABLE 1.

Weekly changes in shell length of juvenile *Haliotis laevis* grown on an *Ulva* germling diet or an *U. lens*/*N. jeffreyi* diet, indicated by the mean increase difference and significance (Univariate Analysis of Variance, post-hoc Tukey HSD tests).

Week Interval	<i>Ulva</i> Germling Diet		<i>U. lens</i> / <i>N. jeffreyi</i> Diet	
	Mean Difference (mm)	Significance (P-Value)	Mean Difference (mm)	Significance (P-Value)
Start-1	0.24	0.904	0.00	1.000
1-2	0.40	0.019	0.40	0.018
2-3	0.67	0.000	0.67	0.000
3-4	0.72	0.000	0.56	0.019
4-5	0.52	0.052	0.80	0.000
5-6	0.44	0.212	0.60	0.009
6-7	0.59	0.010	0.70	0.000
7-8	0.34	0.636	0.44	0.219
8-9	0.41	0.331	0.46	0.151
9-10	0.50	0.085	0.39	0.663
10-11	0.41	0.322	0.66	0.002
11-12	0.47	0.143	0.38	0.461
12-13	0.08	1.000	0.23	0.972
13-14	0.08	1.000	0.25	0.949

#### Abalone Survival

In conjunction with growth, mortality and the subsequent abalone density are important in comparing the two diets effectiveness as a feed for juvenile abalone. Weekly mortality on both diets exhibited very similar profiles with the *U. lens*/*N. jeffreyi* diet producing an average of 91 mortalities in week 3 but thereafter the *Ulva* germling diet recorded slightly higher mortalities until week 10 (Table 3). Calculating the progressive abalone density from the weekly mortality indicated there was no significant difference in abalone density between the two diets ( $F_{(df 1,88)} = 0.569$  ( $P = 0.453$ )). Crushed shells and escapees were unable to be considered in the weekly mortality giving a discrepancy with the final densities. Even though the number of abalone at the end of the 14 wk trial was lower on the *Ulva* germling diet, 1,948 abalone (38.2% survival), compared with the *U. lens*/*N. jeffreyi* diet, 2,390 abalone (46.9% survival), the difference was not significant ( $F_{(df 1,4)} = 3.911$  ( $P = 0.119$ )).

#### Algal Consumption

The juvenile abalone consumed entire *Ulva* germlings, both blade and attachment regions. During the first month of the ex-

TABLE 2.

Weekly growth rates for juvenile *Haliotis laevis* combined into monthly periods ( $n = 3$ ) for both an *Ulva* germling diet and an *U. lens*/*N. jeffreyi* diet. The first 2 weeks were excluded to allow time for the juvenile abalone to adapt to the experimental conditions and diets.

Diet	Week 3-6		Week 7-10		Week 11-14	
	Mean GR ( $\mu\text{m}\cdot\text{day}^{-1}$ )	SE	Mean GR ( $\mu\text{m}\cdot\text{day}^{-1}$ )	SE	Mean GR ( $\mu\text{m}\cdot\text{day}^{-1}$ )	SE
<i>Ulva</i> germling	84.1	9.5	66.1	8.7	35.7	11.8
<i>U. lens</i> / <i>N. jeffreyi</i>	94.1	5.0	68.2	7.5	52.9	11.7

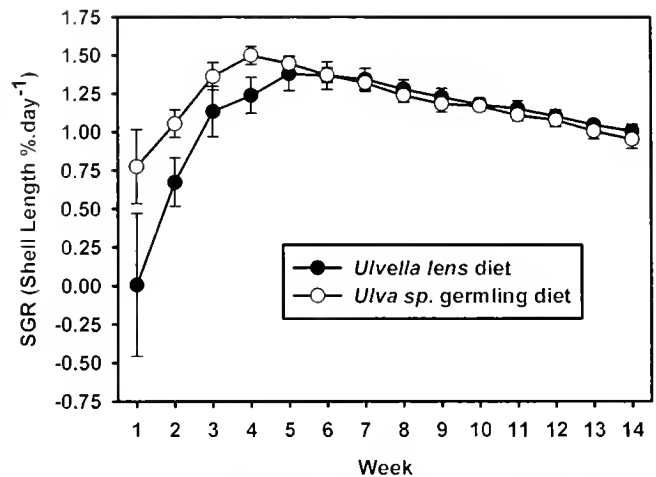


Figure 2. Specific Growth Rate of juvenile *Haliotis laevis* on an *Ulva* germling diet and an *U. lens*/*Navicula* cf. *jeffreyi* diet. Mean  $\pm$  std. error ( $n = 3$ ).

periment, consumption of germlings peaked at 500 germling blades.abalone<sup>-1</sup>.day<sup>-1</sup> but by week 6 the consumption had decreased to 100 germling blades.abalone<sup>-1</sup>.day<sup>-1</sup>. Consumption gradually increased after that (week 6), doubling by the end of the feeding trial (Fig. 3). During the last two months there was a positive correlation between the increase in *Ulva* germling consumption and the increase in abalone shell length ( $R = 0.583$ ;  $P < 0.05$ ).

The consumption of *U. lens* followed a similar trend and was significantly, positively correlated ( $R = 0.422$ ,  $P < 0.05$ ) to the *Ulva* germling consumption but with a reduced rate of decline after the period of high consumption (Fig. 4). Consumption of *U. lens* also significantly correlated to the grow rate ( $R = 0.544$ ,  $P < 0.05$ ) and subsequently the specific growth rate ( $R = 0.618$ ,  $P < 0.05$ ) of the juvenile abalone.

Diatom consumption (Fig. 5) exhibited a similar profile to that of the *Ulva* germlings and *U. lens* consumption including the slow increase after week 7. However this increase fluctuated under or on zero diatoms.abalone<sup>-1</sup>.day<sup>-1</sup> for weeks 7-12 because the positive growth of algae was greater than the consumption by abalone.

TABLE 3.

Weekly mortality of juvenile *Haliotis laevis* for both the *Ulva* germling diet and the *U. lens*/*N. jeffreyi* diet. Mean  $\pm$  std. error ( $n = 3$ ). (Initial number of abalone per replicate tank was 1,700).

Week	<i>U. lens</i> / <i>N. jeffreyi</i>		<i>Ulva</i> Germling	
	Mean	SE	Mean	SE
1	63	5.86	71	5.81
2	81	23.16	85	22.15
3	91	22.4	56	6.44
4	64	14.19	72	12.01
5	53	3.21	57	3.53
6	27	2.91	59	22.21
7	16	3	45	11.35
8	18	2.65	28	2.33
10	24	1.73	25	1.67
12	17	4.63	12	2
14	15	1.76	12	0.88

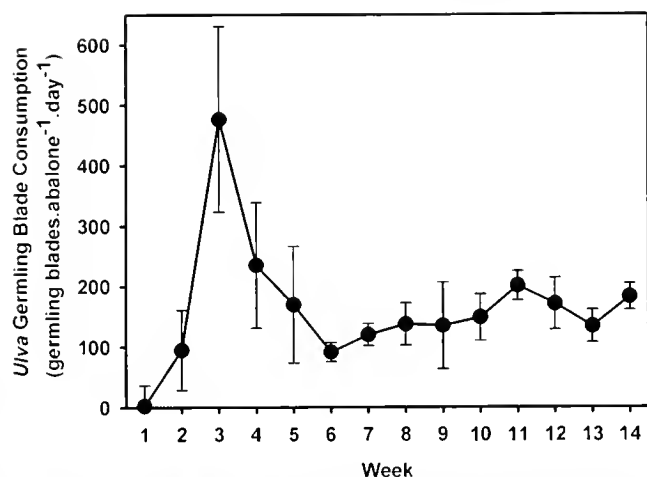


Figure 3. The consumption rate of *Ulva* germling blades by *Haliotis laevis* juveniles over 14 wk (no. of blades.abalone<sup>-1</sup>.day<sup>-1</sup>). Mean  $\pm$  std. error ( $n = 3$ ).

Subsequently the diatom consumption did not correlate with either the *Ulva* germlings or the *U. lens* consumption, nor did it appear to relate to abalone growth.

During the feeding trial *N. jeffreyi* was reinoculated and the PVC plates rotated as illustrated in Table 4 and Figure 5. The consumption of diatoms corresponds to the plate rotation rather than to reinoculation. When comparing the two rotational profiles (1 = start at top - bottom - top and 2 = start at bottom - top - bottom) of diatom consumption they were shown to be statistically different ( $t_{(df=41)} = -2.986$  [ $P = 0.005$ ]). However, if the plates were not rotated then consumption was not significantly different. This indicates that rotating the plates had a considerable effect on the consumption of *N. jeffreyi*.

Two other species of diatoms, *Melosira cf. moniliformis* and *Synedra* sp. contaminated the *Ulva* germling treatment at various stages (Table 4). These diatoms were present throughout the 14 wk, however they only bloomed at the top of the plates on 3 separate occasions (start, week 11 and week 13). The contamination was quickly removed by physically detaching (hand abrasion) it from the substrate then siphoning the tanks' contents.

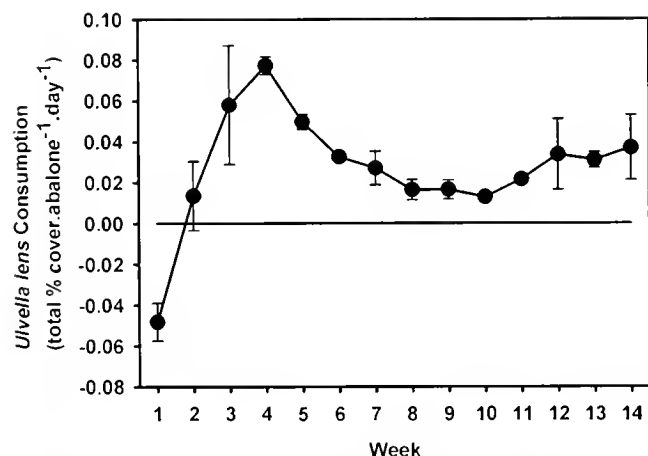


Figure 4. The consumption rate of *Ulvella lens* by juvenile *Haliotis laevis* over 14 wk (% cover.abalone<sup>-1</sup>.day<sup>-1</sup>). Mean  $\pm$  std. error ( $n = 3$ ).

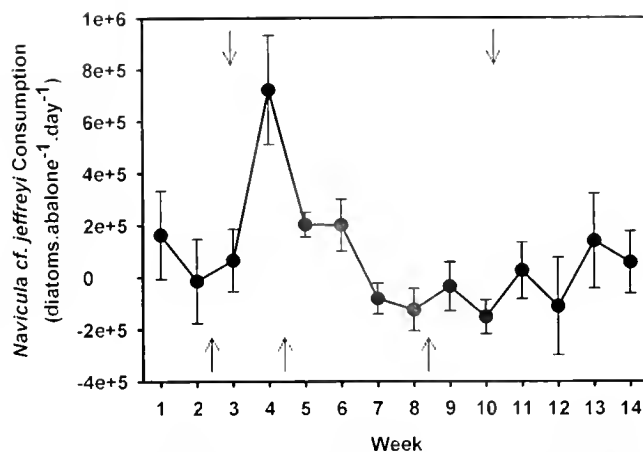


Figure 5. The consumption rate of *Navicula cf. jeffreyi* by *Haliotis laevis* juveniles over 14 wk (diatom cells.abalone<sup>-1</sup>.day<sup>-1</sup>). Mean  $\pm$  std. error ( $n = 3$ ). The arrows at the top indicate plate rotation and the arrows at the bottom indicate inoculation.

#### Biochemical Composition of Algal Diets

The proximate biochemical composition of the *U. lens/N. jeffreyi* diet and *Ulva* germling diet can be seen in Table 5. The two diets both exhibit dry weights of approximately 12.5% with the *U. lens/N. jeffreyi* only slightly higher. Even though the lipid and carbohydrate components were greater in the *Ulva* germling diets the difference was not significant ( $(F_{(df=1,15)} = 2.141$  [ $P = 0.164$ ]) and  $(F_{(df=1,14)} = 1.767$  [ $P = 0.205$ ]) respectively). The protein level in the *Ulva* germling diet however was shown to be significantly higher ( $(F_{(df=1,16)} = 10.893$  [ $P = 0.005$ ])). The total extractable component (sum of protein, lipid and carbohydrate) was higher for the *Ulva* germlings diet.

#### DISCUSSION

The experimental juvenile macroalgae diet of *Ulva* germlings was comparable to the current commercial diet consisting of *Ulvella lens* and *Navicula cf. jeffreyi* for the growth and survival of juvenile *Haliotis laevis*. The two diets demonstrated similar absolute and specific abalone growth rates but the *U. lens/N. jeffreyi* diet produced significantly larger abalone at the completion of the 14 wk feeding trial. Juveniles feeding on the *U. lens/N. jeffreyi* diet reached 10 mm (SL) in less than 13 wk whereas the *Ulva* germling diet produced juveniles of 9.61 mm (SL) at week

TABLE 4.

The weeks, in which inoculation of *N. jeffreyi* occurred, the plates were rotated and when contaminant diatom species were observed, including their relative size.

	1st	2nd	3rd	
Inoculation	Week 2	Week 4	Week 8	
Plates rotated	Week 3	Week 10		
Contamination present				
<i>Melosira cf. moniliformis</i>	Before start	Week 11	Week 13	
<i>Synedra</i> sp.	Before start	Week 11	Week 13	
Contamination size	Cell length	SE	Cell width	SE
	( $\mu\text{m}$ )		( $\mu\text{m}$ )	
<i>Melosira cf. moniliformis</i>	109.5	2.09	8.9	0.29
<i>Synedra</i> sp.	21.5	0.81	19.7	1.42

TABLE 5.

The biochemical composition of both the *Ulva* germling diet and the *U. lens/N. jeffreyi* diet. Values are on dry matter basis and expressed as g/100g dry weight with standard errors in parentheses ( $n = 9$ ).

Diet	Dry Weight	Lipid	Protein	Carbohydrate
<i>Ulva</i> germling	12.44 (0.54)	7.12 (1.55)	32.30 (1.84)	43.86 (5.87)
<i>U. lens/N. jeffreyi</i>	12.82 (0.57)	4.37 (0.95)	24.17 (1.64)	35.85 (2.76)

14. Daume and Ryan (2004) found that for abalone of a similar initial size to the present trial (4 mm SL), it took less than 15 wk to reach 10 mm (SL) on just *U. lens* with a stocking density of approximately 50 animals per plate.

The transition at week 5, between the *Ulva* germling diet and *U. lens/N. jeffreyi* diet, producing significant larger abalone (Fig. 1) indicates that *Ulva* germlings were a more successful diet for *H. laevis* in the range of 3.5–6 mm. The failure to sustain a growth advantage to week 14 indicates better performance of the *U. lens/N. jeffreyi* diet for *H. laevis* in the range of 6–10 mm. The *Ulva* germling diet can therefore be considered an acceptable commercial diet for juvenile abalone (<6 mm) and used either as an alternative or in conjunction with the current commercial diet of *U. lens/N. jeffreyi*.

In the later phase of the trial, *Ulva* germlings were either no longer able to supply the juvenile abalone with specific nutrients or there was not enough biomass. Lack of biomass was an unlikely cause as only 25% of the *Ulva* germlings had been consumed at this point. To overcome either of these problems, freshly seeded plates could be cycled through to maintain a constant supply of new *Ulva* germlings. Daume et al. (2004) utilized this procedure for *U. lens*, which enabled the high initial growth rates of newly settled *Haliotis rubra* to be maintained for 114 d.

The abalone on the *Ulva* germling diet recorded 9 out of the last 10 weekly increases as not significant, which was further compounded by the reduction in abalone weekly growth to only 0.08 mm (SL) for the last 2 wk (Table 1). However the *U. lens/N. jeffreyi* diet also sustained a low weekly abalone growth rate of 0.24 mm (SL) for the last 2 wk. *H. rubra* has been shown to achieve steady growth for 100 d and then fail to grow further on some monospecific algal diets (Day & Fleming 1992).

The reduction of juvenile growth towards the end of the feeding trial occurred at the peak of the winter season with water temperatures dropping from 20.8°C to 19°C. The colder water temperatures may have resulted in the metabolic activity of the abalone reducing, causing less consumption of both algal diets and subsequently slower growth rates.

By week 12 both diets had produced abalone of approximately 9.5 mm (SL) with decreasing growth rates and at this stage could be weaned onto formulated feed (Dunstan et al. 2002, Fleming et al. 1996). However, it could be beneficial to maintain the abalone on its original diet for as long as possible by incorporating fresh seeded plates to reduce competition for food as well as the stress caused by handling (Daume et al. 2004, Fleming 1995).

An alternative to weaning may be to incorporate the *Ulva* germling diet as part of a mixed/rotational diet when it no longer supports adequate growth by itself (Day & Fleming 1992). The consumption of the subsequent algae, rotated through, may account for the deficiencies in the initial diet, in this case *Ulva* germlings (Simpson & Cook 1998). The plate method of feeding

juvenile abalone directs itself to diet rotation or a mixed diet plan whereby plates seeded with different diets can be interspersed throughout the tanks. Simpson and Cook (1998) and Stuart and Brown (1994) demonstrated that *Ulva* sp. as a singular diet produced the lowest abalone growth rates but when presented in a rotational/mixed diet it sustained excellent growth rates.

Growth rates of *H. laevis* fed the *Ulva* germling diet were not significantly different from those produced on the *U. lens/N. jeffreyi* diet (Table 2). The growth rate profile was similar to that obtained by Daume and Ryan (2004) utilizing *U. lens*, where once the first 2 wk had been removed, the next 6 wk recorded 84  $\mu\text{m}\cdot\text{day}^{-1}$  and the final 6 wk 63  $\mu\text{m}\cdot\text{day}^{-1}$ . The growth rates during the first 2 wk were removed from Table 2 as any nutrient deficiency in a diet may be masked by the abalone ability to utilize its own energy stores (Fleming et al. 1996). As the juvenile abalone were taken off an *U. lens*/naturally occurring diatom diet, the weaning process was considered minimal compared with the recommendation of approximately 50 d (Day & Fleming 1992). However it was important to run the feeding trial for as long as possible to detect any effects of nutrient limitation and to determine the capacity of an alga to maintain acceptable abalone growth (Day & Fleming 1992).

The *Ulva* germling diet achieved growth rates of over 100  $\mu\text{m}\cdot\text{day}^{-1}$  during the first 6 wk. This was comparable to *Haliotis discus discus* growth rates attained over a month on a variety of macroalga germlings including *Colpomenia sinuosa*, *Ectocarpus siliculosus* and *Enteromorpha* sp. (Maesako et al. (1984) as cited in Kawamura et al. [1998]). Takami et al. (2003) showed that *Haliotis discus hannai* of approximately 1.8–2.2 mm and 2.8–2.9 mm shell length could reach growth rates of 80 and 100  $\mu\text{m}\cdot\text{day}^{-1}$  respectively on juvenile sporophytes of *Laminaria japonica*.

The specific growth rate reached over 1.3% $\cdot\text{day}^{-1}$  and finished at 1% $\cdot\text{day}^{-1}$  on both diets with no significant difference between them. The *Ulva* germling diet achieved 1.5% $\cdot\text{day}^{-1}$  at week 4 but then exhibited a slow decline. Corazani and Illanes (1998) reported that *H. discus hannai* obtained a higher specific growth rate (0.69% $\cdot\text{day}^{-1}$ ) utilizing adult *Ulva rigida* than other macroalgal diets, whereas *Haliotis rufescens* achieved the lowest specific growth rate. This was comparable to the 0.71% $\cdot\text{day}^{-1}$  achieved by *H. discus hannai* on an *Ulva* sp. (Uki & Watanabe 1992). *Ulva lactuca* has been found to have reasonable dietary value for *Haliotis tuberculata* (1.16% $\cdot\text{day}^{-1}$ ) but significantly lower for *H. discus hannai* (0.75% $\cdot\text{day}^{-1}$ ) (Mai et al. 1996). *Haliotis iris* was only able to achieve 0.1% $\cdot\text{day}^{-1}$  on *U. lactuca* (Stuart & Brown 1994). Simpson and Cook (1998) also found that the suitability of *Ulva* sp., as a feed was dependent on the abalone species.

The *Ulva* sp. being used in the present study was not manipulated through nutrient enrichment during the 14 wk feeding trial. Enriched *Ulva rigida* has been shown to produce growth rates of juvenile *Haliotis roei* comparable to those achieved on the best performing artificial diets (Boarder & Shpigel 2001). Taylor and Tsvetnenko (2004) showed that only 15 mgN $\cdot\text{L}^{-1}$  enriched *U. rigida* produced significantly higher specific growth rates (0.28  $\mu\text{m}\cdot\text{day}^{-1}$ ) than unenriched *U. rigida*. Shpigel et al. (1999) reported growth rates of 44.47 and 121.47  $\mu\text{m}\cdot\text{day}^{-1}$  for *H. discus hannai* and *H. tuberculata* respectively on a high ammonia-N enriched *U. lactuca* compared with 31.7 and 80.72  $\mu\text{m}\cdot\text{day}^{-1}$  on low ammonia-N enriched *U. lactuca*.

The growth rate for *H. tuberculata* produced on the high-enriched *U. lactuca* (Shpigel et al. 1999) was the only growth rate to exceed that obtained on the *Ulva* germling diet in this study.

During the culturing process, before the feeding trial began, the *Ulva* germlings were grown in f/2 media (minus the PII metals, sodium metasilicate and vitamin stock solutions) (Guillard & Ryther 1962). The elevation in nutrients at the start may have led to the extremely high level of consumption (week 3, Fig. 3) resulting in the significantly larger abalone size during the first 5 wk and in turn the rapid  $1.5\% \cdot \text{day}^{-1}$  specific growth rate. Therefore it is important to investigate the benefit of culturing nutrient enriched (high ammonia-N seawater) *Ulva* germlings to achieve the best juvenile abalone growth rates.

The consumption rate of *Ulva* germlings was extremely high during the first month and subsequently the abalone grew rapidly. However, once the consumption rate reduced so did the growth rates giving a significant correlation (Fig. 3). Hone (1992) showed that *Ulva australis* was rapidly consumed by abalone. On a quantitative basis *Ulva* sp. had the lowest consumption in  $\text{g} \cdot \text{abalone}^{-1} \cdot \text{day}^{-1}$  compared with five other adult macroalgae and subsequently produced the lowest growth rates for *Haliotis midae* (Simpson & Cook 1998). Boarder and Shpigel (2001) reported that inorganically enriched *U. rigida* had the lowest consumption rate but was still able to produce growth rates of *H. roei* comparable to that achieved on some of the best artificial diets. As mentioned before, ammonia-N enriched *U. lactuca* produced the highest growth rates for both *H. discus hannai* and *H. tuberculata* but these rates were recorded while consuming significantly less biomass (Shpigel et al. 1999). This indicates that nutrient enriched *Ulva* sp. produces greater growth rates, while requiring less biomass to achieve them.

Daume and Ryan (2004) reported that at the start of a feeding trial *U. lens* had 55% coverage and decreased until 11% was left at 9 wk, when new plates were introduced. That consumption pattern is considerably faster than recorded in this study with 14 wk needed to achieve approximately 11% cover from a similar start value. The different stocking density accounts for the difference in *U. lens* consumption rates with the present study starting approximately 20 fewer animals per plate (Daume & Ryan 2004).

The *U. lens* consumption was significantly correlated with the *Ulva* germling consumption indicating that the juvenile abalone exhibited a similar preference for the two species of green algae (Fig. 4). This is understandable because the biochemical profiles of the two diets were fairly similar (Table 5). The dry weight of both diets, even though similar (12.5%), was lower than  $\approx 15\%$  expected for the majority of algae such as *Ulva* sp. (Mercer et al. 1993, Shpigel et al. 1999). This may be caused by the sampling method incorporating all the biofilm/moisture from the plates rather than just the two green algae.

The *Ulva* germling diet exhibited a higher overall percentage extracted, possibly because of the high ash content of diatoms including *Navicula* (Brown & Jeffrey 1995). The individual biochemical components were also higher. Specifically, the protein level (32.3%) which was significantly larger than that of the *U. lens/N. jeffreyi* diet. It was nearly identical to that of *U. rigida* when enriched from 11.4% to 32.2% protein by using high nutrient water ( $5 \text{ g} \cdot \text{N} \cdot \text{m}^{-2} \cdot \text{day}^{-1}$ ;  $0.6 \text{ g} \cdot \text{P} \cdot \text{m}^{-2} \cdot \text{day}^{-1}$ ) (Boarder & Shpigel 2001). The *Ulva* germlings as a 3-dimensional juvenile macroalgae are in a phase of high growth and therefore may be able to utilize the limited nutrient supply in the water extremely well compared to what the 2-dimensional *U. lens/N. jeffreyi* diet can.

The total percentage extracted ( $\approx 65\%$ ) from the *U. lens/N. jeffreyi* diet may have been reduced because of the combination of microalgae present within the diet. Brown and Jeffrey (1995) ex-

tracted only 56% from *N. jeffreyi* with protein as the major constituent and carbohydrate the lowest, whereas 12% lipid, 28% protein and 7% carbohydrate have been extracted for a combination of diatoms (Brown et al. 1997). The biochemical composition can vary considerably between diatom species let alone a diet containing *U. lens*, diatoms and biofilm components (Brown 1991, Brown et al. 1997, Lewin & Guillard 1963).

Importantly the lower level of lipid, protein and carbohydrate present within the *U. lens/N. jeffreyi* diet produced significantly larger abalone at the end of the 14 wk feeding trial. Therefore the higher amounts of the biochemical components do not increase growth but rather an optimal level may be responsible. Lipid levels of 4% to 5% have been shown to be optimal for abalone, which corresponds with the *U. lens/N. jeffreyi* diet, whereas the *Ulva* germling diet was higher (Dunstan et al. 2000, Uki & Watanabe 1992). High levels ( $\geq 5\%$ ) of dietary lipid have been shown to be detrimental to abalone growth and are believed to depress the digestibility of other nutrients (Britz & Hecht 1997, Uki & Watanabe 1992, Van Barneveld et al. 1998). Therefore the high lipid level present in the *Ulva* germling diet may have restricted the optimal growth rates obtained in the first month of the trial.

Optimal protein levels of 28% have been reported but can range from 20% to 35% depending on abalone species (Britz & Hecht 1997, Coote et al. 2000, Mai et al. 1995, Uki & Watanabe 1992, Vandepeer & Van Barneveld 2002). However to maximize protein utilization not only should the diet contain sufficient readily digestible protein but a well balanced mixture of essential and non-essential amino acids (Britz & Hecht 1997, Mai et al. 1995). Therefore the *U. lens/N. jeffreyi* diet may provide biochemical components closer to the optimal levels for juvenile *H. laevigata* because it produced larger individuals.

The *N. jeffreyi* consumption (Fig. 5) did not correlate with either of the algae species nor any of the abalone results. Weeks 7–10 and 12 all produced negative consumption indicating that the *N. jeffreyi* was reproducing faster than the abalone could consume it. The consumption rate of *N. jeffreyi* did not correspond with the reinoculation but rather the plate rotation  $180^\circ$  about the horizontal.

This can be accounted for by two reasons; firstly because the light gradient through the tanks allowed *N. jeffreyi* situated at the top to receive greater light intensity inducing faster growth and secondly because changes in grazing pressure caused by light sensitivity/migration of abalone. These notions were substantiated through visual observation during the trial because diatom counts increased when at the top and juvenile abalone were found on the bottom of the tanks during the day. Positive relationships between feed intake and the duration of darkness have been shown by Dixon (1992) and Fleming et al. (1996), hence, when the juvenile abalone migrate from the bottom to feed high density of diatoms are closer reducing the effort expended to graze. Daume et al. (2004) indicated that the light intensity tended to be higher at the top of plates and migration to the bottom of the tank was evident.

When the plates were rotated at week 3 the high *N. jeffreyi* densities at the top were transferred to the bottom. This caused the high densities to be closer to the majority of abalone and subsequently caused a spike in consumption (Fig. 5). *N. jeffreyi* consumption dropped as the density at the bottom declined, whereas the density increased at the top because of greater light intensity and less grazing pressure. At week 7 the consumption became negative because the growth at the top exceeded the consumption at the bottom. Once the second rotation at week 10 was performed

the consumption began to increase again as the high *N. jeffreyi* density was available to the juvenile abalone at the bottom. The two rotational profiles (i.e., top-bottom-top vs. bottom-top-bottom) were shown to be significantly different. Therefore it would be beneficial to rotate diatom-cultured plates at least weekly to maintain high diatom densities at the bottom where the majority of abalone reside.

The contaminating diatom species only occurred in the *Ulva* germing diet tanks indicating that it was present from the creation of the diet. It was not determined if the juvenile abalone utilized these contaminating diatoms as a food source although *Synedra* sp. was probably of a suitable size. It may have been difficult for the juveniles to deal with the *Melosira cf. moniliformis* because along with its large cell size it proceeded to rapidly form into dense mats with chains exceeding 5 cm in length. The large blooms of *M. moniliformis* occurring at week 11 and 13 may have had some impact on the declining growth rates because the juveniles are susceptible to smothering and entanglement (Daume et al. 2004).

*Ulva* germings are a suitable feed for juvenile *H. laevigata* because they produced comparable absolute and specific growth rates to the *Ulvella lens* and *Navicula cf. jeffreyi* diet currently used in commercial aquaculture. Further investigation into the theoretical and procedural principles behind the development of the *Ulva* germing diet could allow for the diet to incorporate a variety of different algal species.

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## GROWTH RATE ESTIMATION OF *HEXAPLEX (TRUNCULARIOPSIS) TRUNCULUS* (GASTROPODA: MURICIDAE) BASED ON MARK/RECAPTURE EXPERIMENTS IN THE RIA FORMOSA LAGOON (ALGARVE COAST, SOUTHERN PORTUGAL)

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**ABSTRACT** This study reports growth rates estimates for *Hexaplex (Trunculariopsis) trunculus* (Gastropoda: Muricidae) from mark/recapture experiments carried out in the Ria Formosa lagoon (Algarve coast, southern Portugal). A total of 726 specimens (shell length and total weight ranging between 20.65–58.36 mm and 0.86–19.89 g, respectively) were marked with Dymo tape tags and released into a fish culture earth pond. During the marking process, no adverse effects on the whelks' health and behavior or immediate postmarking mortality were detected. Periodical recapture operations were undertaken using a traditional fishing gear designated locally as "wallet-line" and by hand gathering by scuba divers. A total of 170 whelks were caught, with a total of 216 recaptures (shell length and total weight ranging between 36.22–65.97 mm and 4.42–27.35 g, respectively), thus corresponding to a recapture rate of 29.8%. Despite the loss of one tag, all remaining tags were intact and easily readable. Marked individuals presented low monthly growth rates, in terms of shell length (1.0 mm/month or 2.3%/month), shell perimeter (2.0 mm/month or 3.1%/month) and total weight (0.7 g/month or 10.8%/month), which were highly variable between individuals and higher in smaller specimens. Data were used to estimate the von Bertalanffy growth parameters (length and weight) ( $K=0.41$ ,  $L_{\infty}=82.76$ ,  $W_{\infty}=49.97$  and  $t_0=-0.05$ ). The growth rate of *T. trunculus* was compared with results obtained in similar studies with other gastropod species to evaluate its potential for molluscan aquaculture.

**KEY WORDS:** *Hexaplex (Trunculariopsis) trunculus*, Gastropoda, Muricidae, mark/recapture, growth rate

### INTRODUCTION

Marking methods have been readily applied to several species of gastropods, mainly because of the presence of an external shell on which marks or tags can be attached with little or no adverse effects to the animals (Jones 1979, Gosselin 1993). Such experiments with gastropods have been used in diverse types of studies, to assess movement (Eversole & Anderson 1988, Himmelman 1988), fishing rate (Hancock 1963), field of attraction and effective fishing area of baited traps (Himmelman 1988, McQuinn et al. 1988, Sainte-Marie 1991), species' behavior towards baited traps (Ito et al. 1980), population size and mortality (Hancock 1963) and growth rate (Hancock 1963, Eversole & Anderson 1988).

Various marking techniques have been used in gastropods, such as painting the shell spire with quick drying paints (Hancock & Urquhart 1959, Hancock 1963, Jones 1979, Ito et al. 1980), applying color coded nail polish to the shell (Laxton 1970, Gosselin 1993), attaching rubber bands or nylon lines with tags around the shell (Appeldoorn 1988, Himmelman 1988, McQuinn et al. 1988, Sainte-Marie 1991), gluing a variety of tags to the shell (Hancock & Urquhart 1959, Smith 1987, Eversole & Anderson 1988, Amos & Purcell 2003), scrubbing the shell lip free from the periostracal layer (Hancock & Urquhart 1959, Hancock 1963), drilling and wiring the shell (Laxton 1970, Weil & Laughlin 1984, Kideys & Nash 1993, Kideys 1994), chemically marking the shell and operculum with fluorescent dyes (Kideys & Nash 1993) and using underwater metal detectors to recapture specimens marked with aluminum tags (Crowe et al. 2001).

The muricid gastropod *Hexaplex (Trunculariopsis) trunculus* (Linnaeus, 1758) is a common inhabitant of the subtidal and in-

tertidal areas of the Ria Formosa lagoon (Algarve coast, southern Portugal), where it is subjected to a locally important artisanal fishery. This activity is traditionally undertaken both by manual harvesting during low tide and with an illegal and artisanal fishing gear known as "wallet-line," but more recently this species has also been caught by scuba divers operating illegally inside the lagoon. Furthermore, because of a growing demand for gastropods in the seafood market, the commercial value of *T. trunculus* in Portugal has increased markedly in recent years (reaching prices of €10 – 15/kg for first sale), which has been generating high expectations in terms of its potential as a new species for molluscan aquaculture.

Integrated in a general study on the biology, ecology and fishery of *T. trunculus* in the Ria Formosa lagoon, this work aimed to assess the growth rate of this species by mark/recapture experiments in a fish culture earth pond. To the authors' knowledge, this was the first experiment of this kind ever made with *T. trunculus* and the first growth data (growth rates and growth parameters) available for this species. Moreover, the information gathered in this study will be most useful for assessing the potential of *T. trunculus* for molluscan aquaculture, as well as for establishing adequate management measures for this artisanal fishery.

### MATERIALS AND METHODS

#### Mark/Recapture Experiments

Marking experiments were carried out with commercial samples of *T. trunculus* from the Ria Formosa lagoon (Algarve coast, southern Portugal). Prior to marking, colonizing algae and/or encrusting organisms (mainly polychaetes) were removed from the shell with a hard brush and a small area of the largest whorl of the shell was carefully smoothed with sandstone and clean dried

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with absorbent paper. Subsequently, the specimens were tagged with Dymo tape with two alphanumeric characters. The margins of these tags were cut with scissors, ensuring a tag of  $\approx 6 \times 4$  mm size and facilitating adherence to the rounded and irregular surface of the gastropod shell. The tags were fixed with cyanoacrylate glue and covered with epoxy glue (Fig. 1A), to diminish abrasion of the tag caused by the burrowing movements of the whelks into the sediment and the settlement of encrusting organisms over the tags, which could jeopardize mark retention and readability. The tags were generally fixed between the two most recently deposited growth bands of the last whorl of the shell (Fig. 1A), to prevent eventual dropping of excessive glue from the shell onto the soft body of the gastropods. Finally, after allowing the glue to dry for approximately half hour, marked individuals were rinsed in seawater to avoid potential contamination problems provoked by glue residues, and maintained overnight in an aquarium with running seawater, where they were examined for general condition (comparison of behavior between marked and unmarked specimens).

Mark/recapture experiments were undertaken in a fish culture earth pond of the Olhão fish culture experimental station, which was previously limited by a plastic net fence (area  $\approx 100$  m<sup>2</sup>; maximum depth  $\approx 2$  m). This earth pond receives water directly from the adjacent Ria Formosa lagoon and closely resembles the surrounding natural environment in terms of sediment type, water quality and food availability. Seawater temperature and dissolved oxygen in the earth pond were monitored daily with a multi parameter monitor (Yellow Springs Incorporated, YSI 6820). Periodic recaptures were undertaken monthly, using a traditional fishing gear designated locally as "wallet-line" baited with cockles (*Cerastoderma edule*) and by hand gathering by scuba divers (whenever water visibility allowed for diving recapture operations).

During the marking process and immediately after the recapture operations, specimens were measured for shell length (SL-mm) (Fig. 1B) with a digital calliper (MITUTOYO Digimatic; CD-15D; precision = 0.01 mm), shell perimeter (SP-mm) of the last whorl of the shell (Fig. 1B) and weighed for total weight (TW-g) on a top loading digital balance (AND; HF-2000 G; precision = 0.01 g). Particular care was taken to drain as much water as possible from the mantle cavity before weighing. On both occasions, the position of the tag on the gastropod shell was also registered to verify the degree of shell deposition (tag position at recapture versus tag position at marking) (Fig. 1C).

#### Estimation of Growth Rates

Monthly growth rates in terms of unit/month and percentage/month (mm and %SL/month, mm and %SP/month, g and %TW/month, no. and %growth bands/month) were estimated with the following equations:

$$GR = \frac{(S_r - S_m)}{(t_r - t_m)} \times 30 \quad \text{and} \quad \%GR = \frac{GR}{S_m} \times 100$$

where: GR is the monthly growth rate (unit/month); %GR is the monthly growth rate (%/month);  $S_r$  is the size at recapture (SL-mm, SP-mm, TW-g or tag position);  $S_m$  is the size at marking (SL-mm, SP-mm; TW-g or tag position);  $t_r$  is the day of recapture; and  $t_m$  is the day of marking.

In the case of multiple recaptures, the size increment used (length, perimeter, weight and tag position) was derived from initial size and last recapture.

The relationships between the individuals' size (shell length at marking process, grouped in 5 mm SL classes) and the respective monthly growth rates (unit/month and percentage/month) were assessed by regression analysis (least squares method) and the degrees of association between variables were estimated by the correlation coefficient ( $r$ ).

#### Estimation of Growth Parameters

Whenever the period at liberty (interval between marking and recapture) is small, growth rates may be related to the mean length between marking and recapture ( $(S_m + S_r)/2$ ), using the Gulland-Holt plot (Gulland & Holt 1959). Because the present mark/recapture data lacked small individuals ( $<20$  mm SL), data on growth rates of individuals hatched at the end of June 2004 in the Tavira molluscan aquaculture experimental station were pooled with data on growth rates obtained in mark/recapture experiments. Data were converted into weekly growth rates to increase the number of points in the plot and thus enhance the accuracy of this graphical method. Additionally, recaptured individuals that did not grow during the study period were removed from this estimation of growth parameters.

Growth parameters ( $K$  and  $L_\infty$ ) were estimated from the Gulland-Holt plot by the following relations (Gulland & Holt 1959):

$$K = -b \quad \text{and} \quad L_\infty = \frac{-a}{b}$$

where:  $K$  is the growth coefficient;  $L_\infty$  is the asymptotic shell

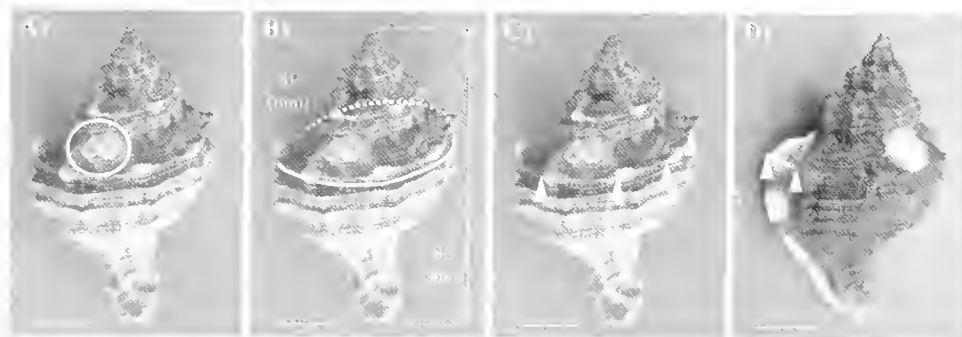


Figure 1. A, *Hexaplex (Trunculariopsis) trunculus* marked with a Dymo tape tag (circle); B, Schematic representation of the shell length (SL-mm) and shell perimeter (SP-mm) measurements; C, Location of the shell growth bands (arrows); D, *T. trunculus* recaptured with a recently deposited growth band (arrows); (scale bar = 10 mm).

length (SL-mm);  $a$  is the linear regression intercept on the Y-axis; and  $b$  is the linear regression slope.

The theoretical age at shell length zero ( $t_0$ ) cannot be obtained from mark/recapture data alone (without specific size-at-age information). For the present purposes  $t_0$  was assumed to correspond to the shell length of *T. trunculus* at hatching ( $L_r = 1.64 \pm 0.22$  mm SL,  $n = 100$ ) (Vasconcelos et al. 2004a) and was calculated from the following expression (von Bertalanffy 1938):

$$t_0 = t + \frac{1}{K} \ln \frac{(L_\infty - L_r)}{L_\infty}$$

Subsequently, shell growth of *T. trunculus* was modeled by applying all these growth parameters ( $K$ ,  $L_\infty$  and  $t_0$ ) in the von Bertalanffy growth in length function, expressed by the following equation (von Bertalanffy 1938):

$$L_t = L_\infty [1 - e^{-K(t-t_0)}]$$

where:  $L_t$  is the shell length at age  $t$  (SL-mm);  $L_\infty$  is the maximum asymptotic shell length (SL-mm);  $K$  is the von Bertalanffy growth coefficient; and  $t_0$  is the theoretical age at shell length zero (years).

After applying the value estimated for maximum asymptotic shell length ( $L_\infty$ ) in the weight/length relationship ( $TW = a * SL^b$ ) for this species, the maximum asymptotic total weight ( $W_\infty$ ) was obtained. Finally, growth in total weight of *T. trunculus* was modeled by the von Bertalanffy growth in weight function, by using the following expression (von Bertalanffy 1938):

$$W_t = W_\infty [1 - e^{-K(t-t_0)^n}]$$

where:  $W_t$  is the total weight at age  $t$  (TW-g);  $W_\infty$  is the maximum asymptotic total weight (TW-g);  $K$  is the von Bertalanffy growth coefficient;  $t_0$  is the theoretical age at total weight zero (years); and  $n$  is the slope ( $b$ ) of the weight/length relationship ( $TW = a * SL^b$ ).

## RESULTS

### Mark/Recapture Experiments

A total of 726 whelks were marked and released between March 2003 and June 2004. These marked specimens had an average shell length of  $44.32 \pm 5.34$  mm and average total weight of  $8.94 \pm 3.06$  g (ranges 20.65–58.36 mm and 0.86–19.89 g, respectively). A careful inspection of the condition of marked individuals held in the aquarium did not reveal any adverse effects on the gastropods health and behavior or immediate postmarking mortality.

Daily monitoring of temperature and dissolved  $O_2$  in the earth pond (Fig. 2) showed that the average temperature was relatively high ( $20.7 \pm 4.9^\circ\text{C}$ ), varying between a minimum of  $11.0^\circ\text{C}$  in December 2003 and a maximum of  $29.4^\circ\text{C}$  in August 2003. The average dissolved  $O_2$  was  $7.2 \pm 1.1$  mg/L, showing an opposite trend relative to water temperature, with a maximum in May 2004

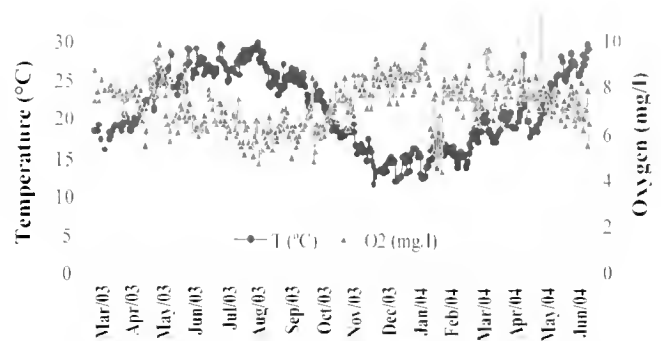


Figure 2. Seawater temperature and dissolved oxygen in the earth pond where the marked specimens of *Hexaplex (Trunculariopsis) trunculus* were released.

( $11.4$  mg/L) and an unexpected minimum in February 2004 ( $4.2$  mg/L).

In the mark/recapture study, 170 whelks were caught (216 recaptures) with an average shell length of  $49.36 \pm 4.92$  mm and average total weight of  $11.89 \pm 4.08$  g (ranges 36.22–65.97 mm and 4.42–27.35 g, respectively). Overall, these 216 recaptures corresponded to a recapture rate of 29.8%. In this context, it is worth emphasizing that most recaptures were caught only once (133 individuals = 78.2%), 30 whelks were recaptured twice (17.7%), six were caught three times (3.5%), and one was recaptured four times (0.6%). In all, 107 dead whelks were recovered from the earth pond during diving operations, corresponding to a mortality rate of 14.7%. After 16 mo from the beginning of the study, only one specimen was recaptured without a tag. All other recaptured specimens had their tags intact and easily readable, most of them presenting recently deposited growth bands (Fig. 1D).

### Estimation of Growth Rates

The recaptured whelks presented average monthly growth rates of  $1.0 \pm 1.0$  mm SL ( $2.3 \pm 2.6\%$  SL/month),  $2.0 \pm 1.6$  mm SP ( $3.1 \pm 2.8\%$  SP/month) and  $0.7 \pm 0.6$  g TW ( $10.8 \pm 11.3\%$  TW/month). However, growth rates were highly variable among individuals, with some whelks simply not growing (in terms of shell length, shell perimeter and/or total weight), whereas the fastest growing specimens exhibited much higher growth rates i.e., 4.0 mm SL/month ( $13.4\%$  SL/month), 7.4 mm SP/month ( $15.7\%$  SP/month) or 3.4 g TW/month ( $59.0\%$  TW/month) (Table 1).

Individually, growth rates varied with size (initial shell length), being highly variable between individuals, and generally higher in smaller than in larger individuals. In fact, individual monthly growth rates displayed a decreasing trend during ontogeny, a phenomenon that was more evident in the relationships between growth in shell length, shell perimeter and shell deposition as a function of shell length (Figs. 3A, B, C, D, G, H), than in the rela-

TABLE 1.

Monthly growth rates (shell length, shell perimeter and total weight) of the *Hexaplex (Trunculariopsis) trunculus* specimens recaptured in the earth pond.

Monthly Growth Rate (N = 216)	Shell Length (SL-mm)		Shell Perimeter (SP-mm)		Total Weight (TW-g)	
	mm/month	%/month	mm/month	%/month	g/month	%/month
Mean $\pm$ SD	$1.0 \pm 1.0$	$2.3 \pm 2.6$	$2.0 \pm 1.6$	$3.1 \pm 2.8$	$0.7 \pm 0.6$	$10.8 \pm 11.3$
(Min.–Max.)	(0.0–4.0)	(0.0–13.4)	(0.0–7.4)	(0.0–15.7)	(0.0–3.4)	(0.0–59.0)

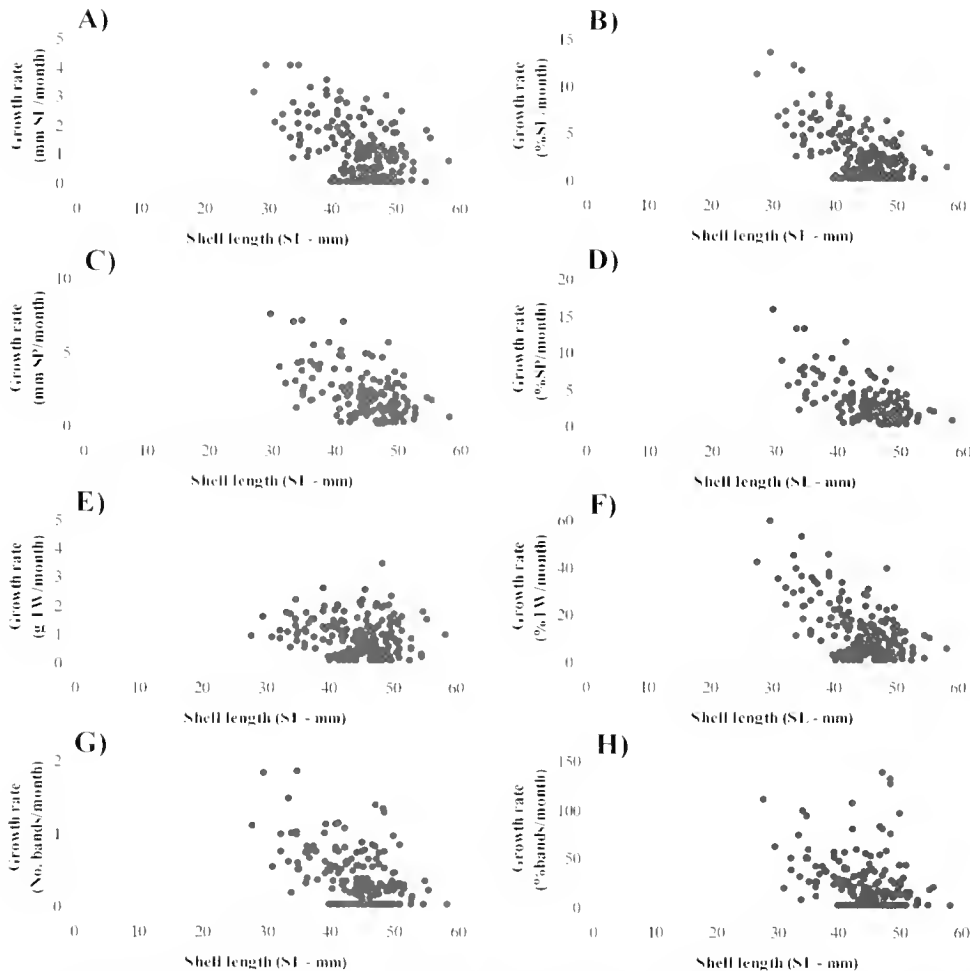


Figure 3. Individual monthly growth rates of recaptured specimens of *Hexaplex (Trunculariopsis) trunculus* in terms of shell length, shell perimeter, total weight and shell deposition: A, SL/month; B, %SL/month; C, SP/month; D, %SP/month; E, TW/month; F, %TW/month; G, no. growth bands/month; H, %growth bands/month.

tionships between growth in total weight in function of shell length (Figs. 3E, F).

Because of the high interindividual variability observed in monthly growth rates, individual data were grouped in size classes (5 mm SL) and subjected to regression analyses (Fig. 4). After pooling data into size classes, the inverse relationship between size (shell length at marking) and growth rates became even more evident, further strengthening the decreasing trend in growth during ontogeny. This was particularly evident in the relationships SL/month in function of shell length (Figs. 4A, B) and SP/month in function of shell length (Figs. 4C, D), whereas the relationships TW/month in function of shell length (Figs. 4E, F) presented poorer fittings. Furthermore, the relationships established between shell deposition rate (growth bands/month) and shell length (Figs. 4G, H) perfectly illustrate the remarkable decline in shell deposition with specimen size (i.e., during growth), reflected by the high correlation coefficients ( $r$ ) registered in these regressions.

#### Estimation of Growth Parameters

The Gulland-Holt plot of weekly growth rate versus mean shell length (with pooled data from mark/recaptured gastropods and from laboratory hatched gastropods) is illustrated in Figure 5A. After applying this graphical method, the estimated growth param-

eters were  $K = 0.008/\text{wk}$  (0.41/y) and  $L_{\infty} = 82.76$  mm SL. The calculation of  $t_0$ , assuming the shell length of *T. trunculus* at hatching of  $L_t = 1.64$  mm SL, produced a value of  $-0.05$  y. The von Bertalanffy growth function for *T. trunculus* in terms of shell length ( $L_t = 82.76[1 - e^{-0.41(t+0.05)}]$ ) is presented in Figure 5B.

After employing the weight-length relationship ( $TW = 0.00008SL^{3.022}$ ;  $n = 2401$ ;  $r = 0.975$ ;  $P < 0.05$ ) estimated by Vasconcelos et al. (2004b) for *T. trunculus* from the Ria Formosa lagoon (Fig. 5C), the von Bertalanffy growth function for this species in terms of total weight ( $W_t = 49.97[1 - e^{-0.41(t+0.05)}]^{3.022}$ ) was obtained (Fig. 5D).

## DISCUSSION

#### Mark/Recapture Experiments

Marked gastropods are frequently disturbed and stressed by tagging procedures (Himmelman 1988) that may result in quite different behavior from undisturbed specimens (Sainte-Marie 1991), often exhibiting a period of stress-induced inactivity during release after extended tagging manipulations (McQuinn et al. 1988). In this study, the marking process had no detectable adverse effects on the marked whelks' health and behavior. When returned to the aquarium, marked whelks soon started crawling (within a

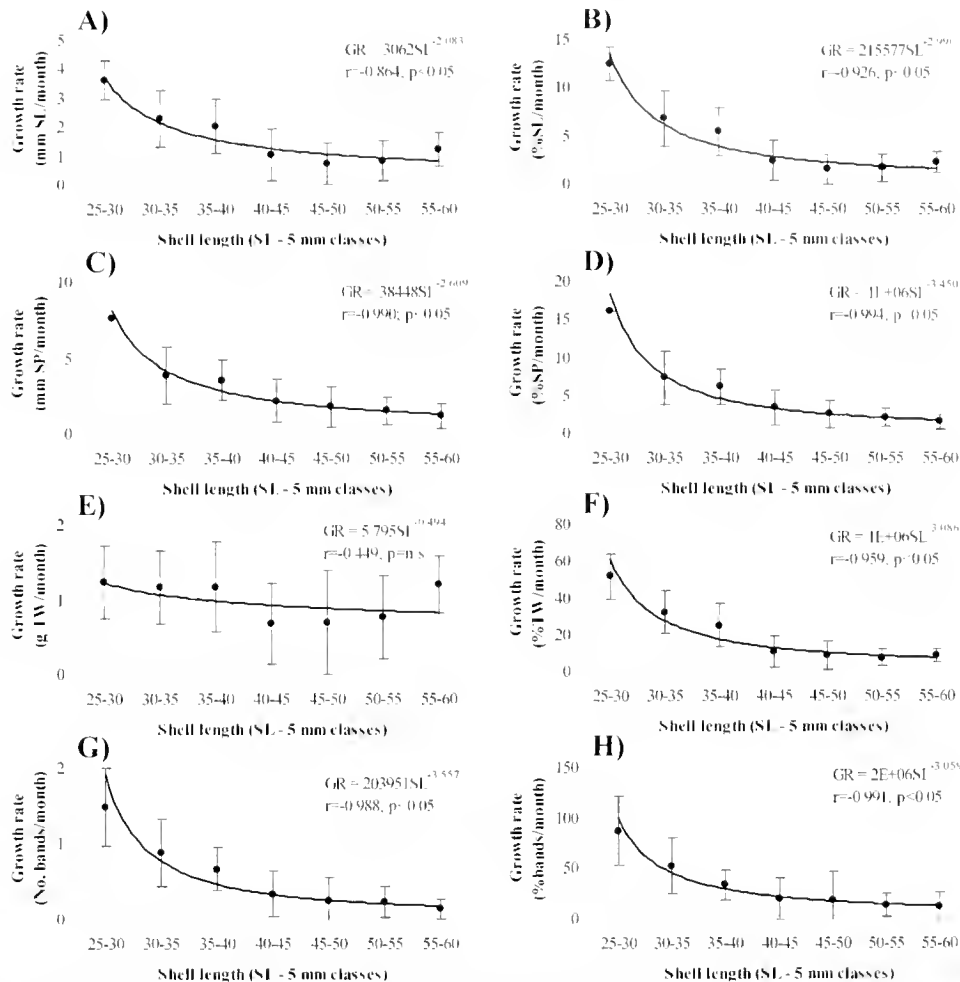


Figure 4. Monthly growth rates of recaptured specimens of *Hexaplex (Trunculariopsis) trunculus* (grouped in 5 mm SL classes) in terms of shell length, shell perimeter, total weight and shell deposition: A, SL/month; B, %SL/month; C, SP/month; D, %SP/month; E, TW/month; F, %TW/month; G, no. growth bands/month; H, % growth bands/month.

maximum of 5–10 min), and no behavioral differences were apparent between marked and unmarked individuals. This was probably because *T. trunculus* quickly withdraws into the shell when removed from seawater, preserving most of the inner moisture.

The recapture rate obtained during these experiments (29.8%) was low, considering that specimens were released inside a fenced area on an earth pond, virtually without potential predators. In this case, the high proportion of uncaught individuals might have been because of the type of bottom in the earth pond (muddy sediment), which favored the burrowing movements of *T. trunculus* and decreased underwater visibility during recapture operations by hand gathering by scuba divers.

An unexpectedly high mortality rate was registered (14.7%). Besides natural mortality, this might have been caused by other circumstances, namely a possible weakness and fragility of some marked individuals. Indeed, the fact that specimens were obtained from commercial samples meant that neither date of capture nor the conditions under which captured whelks were held were known. Additionally, despite water temperature in the earth pond closely reflecting the environmental conditions in the adjacent Ria Formosa lagoon, dissolved oxygen was low during some periods

and might have also contributed to the mortality of marked specimens.

However, this high mortality rate is probably slightly underestimated, because dead individuals could only be recovered from the earth pond bottom by diving, frequently in poor visibility. Moreover, the burrowing behavior of *T. trunculus*, coupled with the high sedimentation rate inside the earth pond, might have also made difficult the detection of all dead specimens, thus probably contributing to an underestimation of total mortality rate.

#### Estimation of Growth Rates

The monthly growth rates estimated in this study, in terms of shell length (1.0 mm/month or 2.3%/month), shell perimeter (2.0 mm/month or 3.1%/month) and total weight (0.7 g/month or 10.8%/month) were considered somewhat low, taking into consideration that *T. trunculus* is supposed to be a species with a short/medium life-span. Nevertheless, this growth rate is common among muricid species, because according to Spight et al. (1974) a typical young muricid grows only 1–2 mm/month. As expected, monthly growth rates decreased markedly during ontogeny, with smaller specimens growing more rapidly than larger ones. This was particularly evident in growth in terms of shell length, shell

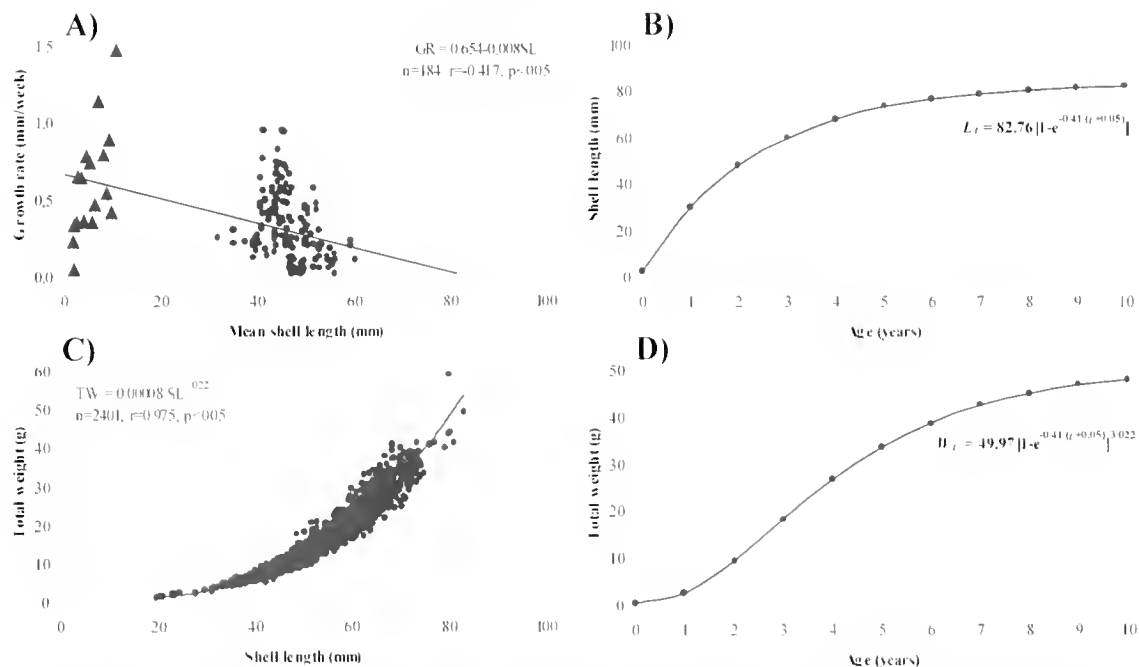


Figure 5. A, Gulland-Holt plot of weekly growth rate versus mean shell length for *Hexaplex (Trunculariopsis) trunculus* (● data from marked/recaptured gastropods, ▲ data from laboratory hatched gastropods); B, von Bertalanffy growth function in terms of shell length for *T. trunculus*; C, weight-length relationship for *T. trunculus* (data from Vasconcelos et al. 2004b); D, von Bertalanffy growth function in terms of total weight for *T. trunculus*.

perimeter and shell deposition rates, but less noticeable in terms of total weight growth rate. This phenomenon was probably influenced by the seasonal fluctuations in physiological condition and total weight normally associated with the reproductive cycle of the species, being accentuated by the fact that it was virtually impossible to assure the complete removal of water from the shells, thus contributing to some variability in weighing both at marking and recapture operations.

Moreover, monthly growth rates were highly variable among individuals and some whelks did not grow between marking and recapture. The same high interindividual variability in growth rates has been noted in several gastropod species and has been attributed to diverse factors (e.g., Tallmark 1980, Smith 1987 and references therein). In fact, most muricid adults simply do not grow (Spight et al. 1974), and in some experiments with different species, larger specimens (presumably adults) did not grow at all during the study period, after becoming reproductively active, even though they were substantially smaller than their maximum shell length (e.g., Hancock 1959, Laxton 1970, Fotheringham 1971, Spight et al. 1974, Tallmark 1980, Appeldoorn 1988). However, in the present case, despite reduction or even cessation of growth in shell length, *T. trunculus* continued slowly growing in shell perimeter, total weight and shell deposition, with monthly growth rates that were invariably higher than shell length growth rates.

However, these growth estimates are most likely underestimated, because despite the inclusion of some juveniles in the experiments, marked and recaptured gastropods were dominated by adult specimens (consequently with a much lower growth rate), which obviously induced a significant underestimation of the overall growth rate of the species. Furthermore, notwithstanding the use of a non size-selective fishing gear in the recapture operations, the samples caught through snorkeling and scuba diving could be size-biased, because bigger whelks were more conspicuous and

more easily detected than smaller individuals, especially in a burrowing species and under low water visibility conditions. In addition, many animals were recaptured less than 1 year after being marked and during winter and spring, when lower water temperatures and less food availability in the earth-pond might have slowed their metabolism and decreased growth rates. For all these reasons, the present growth rates of *T. trunculus* should be interpreted with some caution and considered as average values for the size range used in this study (dominated by adults). Extrapolation of the data to juveniles is especially problematic. *T. trunculus* individuals hatched at the end of June 2004 and maintained until now in the Tavira molluscan aquaculture experimental station presented a much higher growth rate, hatching with  $1.64 \pm 0.22$  mm SL and reaching  $11.66 \pm 2.05$  mm SL after 4 mo (end of October 2004), therefore corresponding to a monthly growth rate of 2.5 mm SL/month during the first 4 mo of life (Vasconcelos et al. 2004a).

The comparison of the growth rates of *T. trunculus* obtained in this study with those obtained for other gastropod species (namely some muricids with high commercial value) was difficult, because most studies had different objectives, different experimental designs (e.g., field vs. laboratory, with vs. without additional food supply) and variable size ranges of the individuals used for growth rate estimations (e.g., juveniles vs. adults) (Table 2). The average monthly growth rate of *T. trunculus* (1.0 mm/month) compared favorably with those obtained for *Ocenebra lurida*, *Urosalpinx cinerea*, *Busycon carica* and *Turbo sarnaticus*, being equal to other growth rates estimated for *B. carica*, *Busycon canaliculatum* and *Ocenebra interfossa*. In contrast, the present growth rate was lower than those obtained for *B. carica*, *Cerastostoma foliatum* and *Cerithium nodulosum* and much lower than the growth rates of *Ocenebra lunaria*, *Eupleura caudata*, *Bolinus brandaris*, *Concholepas concholepas*, *Chicoreus virgineus*, *Strombus gigas* and *Chicoreus ramosus*. In this context, it is worth emphasizing that

TABLE 2.

Comparison of the growth rate of *Hexaplex (Trunculariopsis) trunculus* obtained through mark/recapture experiments with growth rates reported for other gastropod species.

Species	Location	Size range (SL-mm)	Growth rate (SL-mm)	Author(s)
<i>Hexaplex trunculus</i>	Ria Formosa, Portugal	20–58 mm	1.0 mm/month	present study
<i>Ocenebra lurida</i>	Washington, U.S.A.	12–22 mm	0.4 mm/month	Spight et al. (1974)
<i>Busycon canaliculatum</i>	South Carolina, U.S.A.	—	1.0 mm/month	Eversole and Anderson (1988)
<i>Busycon carica</i>	South Carolina, U.S.A.	—	1.0 mm/month	Eversole and Anderson (1988)
<i>Ocenebra interfossa</i>	Washington, U.S.A.	12–21 mm	1.0 mm/month	Spight et al. (1974)
<i>Cerastostoma foliatum</i>	Washington, U.S.A.	juveniles	<2.0 mm/month	Spight et al. (1974)
<i>Cerithium nodulosum</i>	Guam, Mariana Islands	25–75 mm (juveniles)	2.3–4.6 mm/month	Yamaguchi (1977)
<i>Ocenebra lumaria</i>	Asamushi, Japan	—	3.7 mm/month	Luckens (1970)
<i>Eupleura caudata</i>	Virginia, U.S.A.	—	4.0 mm/month	Mackenzie (1961)
<i>Bolinus brandaris</i>	Catalonia, Spain	>5 mm (juveniles)	4.3 mm/month	Ramón and Flos (2001)
<i>Concholepas concholepas</i>	Chile	juveniles	4.7 mm/month	Lara and Montes (1988)
<i>Chicoreus virgineus</i>	Cuddalore, India	70–85 mm	3.0–5.0 mm/month	Ramesh et al. (1992)
<i>Strombus gigas</i>	Los Roque, Venezuela	30–190 mm	4.0–15.0 mm/month	Weil and Laughlin (1984)
<i>Chicoreus ramosus</i>	Phuket, Thailand	25–45 mm (juveniles)	11.4 mm/month	Bech (1992)
<i>Urosalpinx cinerea</i>	England, U.K.	juveniles	3.0 mm/year	Hancock (1959)
<i>Busycon carica</i>	North Carolina, U.S.A.	juveniles	10.2 mm/year	Magalhães (1948)
<i>Turbo sarmaticus</i>	Port Alfred, South Africa	42 mm (juveniles)	2.4–13.8 mm/year	Foster et al. (1999)
<i>Busycon carica</i>	Virginia, U.S.A.	juveniles	13.2 mm/year	Kraeuter et al. (1989)

most of these studies used newly hatched specimens or small juveniles during growth studies, thus growth rates are presumably overestimated considering the overall size ranges (juveniles + adults) of the natural populations of these species. Moreover, some of these species have a maximum size much bigger than *T. trunculus*. Comparisons of growth rates (mm SL/month) between gastropod species are complicated and should be carefully interpreted.

#### Estimation of Growth Parameters

The von Bertalanffy growth function in terms of shell length estimated for *T. trunculus* ( $L_t = 82.76[1 - e^{-0.41(t+0.05)}]$ ) further confirmed that the growth of this gastropod species is relatively slow ( $K = 0.41$ ). According to these data the minimum landing size of *T. trunculus* (MLS = 50 mm SL) is only attained at 2.20 y old. Furthermore, at 95% of its theoretical maximum shell length (95%  $L_\infty = 82.76$  mm), the von Bertalanffy growth equation yielded an estimated age of 7.24 y for *T. trunculus*, which can be considered a relatively short/medium life span for a gastropod species.

The estimated maximum asymptotic shell length ( $L_\infty = 82.76$  mm) closely approached the maximum size attained locally by *T. trunculus*. During a general study on the biology, ecology and fishery of this species in the Ria Formosa lagoon, the largest specimen sampled measured 82.84 mm. Nevertheless, specimens of this size were exceptional, because among 2484 *T. trunculus* measured during a 1-y sampling program, only one individual was bigger than the asymptotic shell length estimated in this study ( $L_\infty = 82.76$  mm), which was very similar to the maximum size attained by this species in the Ria Formosa lagoon (85 mm SL) (Muzavor & Morenito 1999) and included in the maximum size range reported for *T. trunculus* by other authors, between 80 mm SL (Pope & Goto 1991) and 90 mm SL (Macedo et al. 1999).

The von Bertalanffy growth function in terms of total weight estimated for *T. trunculus* ( $W_t = 49.97[1 - e^{-0.41(t+0.05)}]^{3.022}$ ) indicated that at 2.20 y old (age corresponding to the minimum landing

size of *T. trunculus* – MLS = 50 mm SL) specimens achieved 10.86 g in total weight. The estimated maximum asymptotic total weight ( $W_\infty = 49.97$  g) was lower than the maximum total weight registered in samples from the Ria Formosa lagoon (58.20 g), but yet again this was an exceptionally heavy individual, because amongst 2401 *T. trunculus* weighed during a 1-y sampling program, only one specimen was heavier than the asymptotic total weight estimated in the present study ( $W_\infty = 49.97$  g).

Therefore, and although the von Bertalanffy growth model has been criticized for not adequately representing growth of the entire size range of organisms, in the present case both size (shell length) and weight (total weight) data fitted the growth model satisfactorily.

#### Aquaculture and Fisheries Considerations

Despite being slightly low and underestimated because of the dominance of adult specimens in the samples, the growth rate estimated through mark/recapture experiments for *T. trunculus* (1.0 mm/month) may still create some expectations in terms of the potential of this species for molluscan aquaculture, considering that laboratory hatched specimens grow at a much higher rate (2.5 mm/month during the first 4 mo of life) and that the von Bertalanffy growth function indicated an age of 2.20 y to attain the minimum landing size for this species (50 mm SL i.e., 10.86 g TW).

Because environmental and feeding requirements of *T. trunculus* are not demanding, the culture of this gastropod species could become profitable in the near future, when the crucial phases of the species reproductive cycle (spawning and posthatching development) are better known. Moreover, because of an increasing demand for gastropods in the seafood market, the commercial value of *T. trunculus* has been continuously rising (reaching values of €10–15/kg for first sale) and its abundance in the Ria Formosa lagoon apparently has been decreasing over the last years, prob-

ably caused by illegal fishing and consequent overexploitation of the resource.

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## **ABSTRACTS OF PAPERS**

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**ECONOMIC IMPACT ASSOCIATED WITH THE RECREATIONAL SCALLOP SEASON IN CITRUS COUNTY, FLORIDA, USA.**

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Because declining stock conditions, the commercial harvest of bay scallops (*Argopecten irradians*) in Florida was banned during 1994. During that same year, a recreational season was established on the Gulf coast of Florida in the area between Aripeka and Mexico Beach, Florida. In 1995, the recreational season was limited to a more restricted region—the area north and west of the Suwannee River. Because of a number of factors, including efforts to re-establish bay scallops through stock enhancement activities, the recreational season was re-opened during 2002 in the region between Aripeka and the Suwannee River. Within this region, the highest concentration of scallops, and thus recreational scalloping activities, is near the mouths of the Homosassa River and the Crystal River, in Citrus County. The recreational scallop season runs from July 1 to September 10 of each year. During this period, local businesses experience increase business volumes because recreational scallopers arrive in large numbers attempting to harvest daily bag limits. Anecdotal evidence and reports suggest that the recreational scallop season provides a significant boost to the local economy, because recreational scallopers purchase fuel, food, supplies and lodging accommodations during their stay. In addition, many of these recreational scallopers come from outside the area, thereby bringing in new dollars to the local economy. The purpose of this study is to estimate the economic impact associated with the 2003 recreational scallop season in Citrus County, Florida. This information would help resource managers and county business representatives better understand the economic and social implications of ensuring a sustainable recreational harvest of bay scallops. Several personal interviews were conducted immediately after the 2003 season. A total of 99 interviews were conducted. The interviews were conducted with representatives of a variety of business types in the area. The business types included fishing guides and dive shops, restaurants, lodging accommodations, convenience stores and gas stations and retail shops. Sales information associated with clientele involved in recreational scalloping was solicited. Estimates of changes in business sales during the scallop season were requested. Information on the resident versus non-resident sales was sought. The sample survey findings were then extrapolated to the total business population in the region. As a result, the increase in business sales activity during the 2003 recreational scallop season was estimated to be \$1.4 million. Of this amount, approximately \$900,000 was associated with non-resident expenditures. An estimate of economic impact associated with the 2003 scallop season was then developed. The changes in business activity were entered into a regional economic modeling package, IMPLAN Pro. This model provides estimates of the economic impacts associated with changes in business activity within a region. This impact includes the “multiplier” effects that recre-

ational scalloping-related expenditures have on the local economy. As a result, the total economic activity associated with non-resident expenditures on recreational scalloping activities during 2003 in Citrus County, Florida was \$1.6 million. Local incomes increased by \$636,000, and local business taxes increased by \$110,000 because of these non-resident expenditures. Approximately 35 new jobs are associated with the recreational scallop season. Thus, the recreational scallop season provides economic impact to the regional economy. Further research is needed to better refine the economic impact estimates by business type and to expand the analysis into other regions of the state where the recreational scallop season occurs.

**SWIMMING BEHAVIOR OF *PECTEN MAXIMUS* LARVAE.** Sissel Andersen, Anne Berit Skiftesvik and Howard Browman. Institute of Marine Research, Austevoll, N-5392 Storebo, Norway.

Institute of Marine Research (IMR) is working to support the development of a sustainable aquaculture in Norway and great scallop (*Pecten maximus*) is one of the marine species on focus. IMR has worked for several years to improve the results for the production of great scallop spat. As part of a project to investigate several factors on larval performance, larval swimming behavior was observed using Silhouette (shadow) video photography. The factors larval stocking density, algal concentration, and light intensity were investigated at different larval ages. Factors affecting swimming behavior are likely to be important for the energy budget. Batches of scallop larvae were transferred from the bivalve hatchery Scalpro AS 3 days after spawning, and placed in 100–300-L stock tanks with flow-through of seawater and algae. Rearing methods were those employed for flow-through systems at IMR-Austevoll: temperature of 16°C to 17°C, flow rate of 1 tank volume per day, continuous room light, continuous addition of a mixture of three algal species (*Isochrysis galbana*, Tahitian), *Pavlova lutheri* and *Chaetoceros mulleri* mixed in the ratio 1:1:1, algal concentration in rearing tanks at 10 cells/uL and an initial stocking density of 5–8 larvae/mL. Larvae were starved 12 to 24 hours prior to experimental treatment and observations. Silhouette (shadow) video photography (SVP) was used to record the behavioral responses of scallop larvae. Image quality is unaffected by ambient light-level silhouettes are cast equally well under full sun or in complete darkness. It allows filming of events in a large depth of field (~15 cm), with a relatively large field of view (here ~15 cm). The illumination system consisted of 2 components: a light intensity-controlled 1000-W Xenon arc lamp connected to an ultraviolet (UV, 280–400 nm)-visible liquid light guide and a 200-W Xenon arc lamp. Larval swimming behavior was affected by all three factors investigated. Also, the ratio of larvae exhibiting the clock-wise helical swimming pattern at an early stage reflected the quality of the larval batch in terms of survival. The effect on swimming behavior increased as a function of exposure time from 3 minutes up to 4 hours.

**FLOW-THROUGH LARVAL SYSTEMS WITH DIFFERENT ALGAL CONCENTRATIONS.** Sissel Andersen,<sup>1</sup> Gyda Christophersen<sup>2</sup> and Thorolf Magnesen.<sup>2</sup> <sup>1</sup>Institute of Marine Research, Austevoll, N-5392 Storebo, Norway; <sup>2</sup>Centre for Studies of Environment and Resources, University of Bergen, PO Box 7800, 5020 Bergen, Norway

Institute of Marine Research (IMR) is working to support the development of a sustainable aquaculture in Norway. Great scallop (*Pecten maximus*) is one of the marine species being focused on in developing the aquaculture industry. IMR has worked together with the bivalve hatchery Scalpro AS and the University of Bergen for several years to improve the results for the production of great scallop spat. As part of a project to investigate several factors on larval performance in production scale units, larvae were grown with different algal concentration in the rearing tanks. Larval groups were transferred 3 days after spawning from Scalpro AS to large-scale facilities at Austevoll, IMR. The larvae were kept in 2,800 litres up-welling cylindrical tanks with conical bottom. Standard rearing method was used: a flow rate of one tank volume per day, temperature 15°C to 17°C and a diet consisting of *Isochrysis galbana* (Tahitian), *Pavlova lutheri* and *Chaetoceros mulleri* mixed in the ratio 1:1:1. The initial larval stocking density was 3–5 larvae/mL. The variation between tanks was first tested keeping the algal concentration in the larval rearing tanks at 7 ( $\pm 3$ ) algal cells/ $\mu$ L, and then larvae were reared keeping the algal concentration between 3 and 20 cells/ $\mu$ L. Also, one larval group was starved and not supplied with algae. Larval growth and survival was registered, as was the yield of competent larvae retaining on a 150  $\mu$ m mesh screen. The variation between tanks was low. There was no significant difference between tanks in mean shell height 21 days after spawning or larval yield on 150  $\mu$ m mesh 24 days after spawning. Mean shell height ranged between 162  $\pm$  26 and 170  $\pm$  25  $\mu$ m for larvae in the different tanks. Larval yield ranged between 27.4 and 34.8%. Algal concentration in the larval rearing tanks affected both growth rate and metamorphosis rate. Growth of starved larvae ceased 6 days after spawning. There were no significant differences in shell height between fed groups up to 13 days after spawning when the mean shell height was 134  $\pm$  10  $\mu$ m. Later there was a positive correlation between algal concentration and shell growth rate. However, there was no significant difference in shell growth between the two highest algal concentrations (16 and 20 cells/ $\mu$ L), and between the two lowest algal concentrations (3 and 8 cells/ $\mu$ L). Mean daily shell growth at 3 cells/ $\mu$ L was 23% lower than at 20 cells/ $\mu$ L. Generally survival 20 days after spawning was not significantly different between fed groups and varied between 85 and 96%. Survival in the starved groups was similar to fed groups 15 days after spawning but decreased rapidly to 9% 20 days after spawning. Larvae were harvested on a 150- $\mu$ m mesh screen and transferred to a production settlement system in the hatchery. Generally the yield of pediveligers on 150- $\mu$ m mesh screen increased with increasing algal concentration. Time to first harvest was shortest for larval groups

with the two highest algal concentrations: 22 days after spawning. The groups with the two lowest algal concentrations were harvested 27 days after spawning. Yield of pediveligers at first harvest varied from 24% to 48%, the highest value representing the highest algal concentration.

**THE SOUTH AFRICAN SCALLOP *PECTEN SULCICOSTATUS*—PRELIMINARY RESULTS FROM INVESTIGATIONS OF REPRODUCTIVE CYCLE.** Dale C. Z. Arendse,<sup>1</sup> Sissel Andersen,<sup>2</sup> Norman J. Blake<sup>3</sup> and Grant C. Pitcher.<sup>1</sup> <sup>1</sup>Department Environmental Affairs and Tourism, Marine and Coastal Management, Private Bag X2, Rogge Bay, 8012, Cape Town, South Africa; <sup>2</sup>Institute of Marine Research-Austevoll, N-5392 Storebo, Norway; <sup>3</sup>College of Marine Science, University of South Florida, 140 7th Ave. South, St. Petersburg, FL 33701, USA

The local scallop *Pecten sulcicostatus* is presently being investigated as a potential species for mariculture in South Africa. As there is no commercial fishery for scallops in South Africa little is known about the ecology of the local species. Therefore this study was conducted to determine the reproductive cycle of *P. sulcicostatus*. Scallops were collected on a monthly basis in False Bay to calculate the gonadal somatic index (GSI) and to conduct histological studies on the gonads. The study began in August 2004 as a part of a Norwegian-South African cooperation funded by NORAD. Two size classes were collected, 40 mm to 59 mm and 60 mm to 110 mm. A steady decrease in the GSI took place for both size classes from August (winter) to November (spring) 2004. The GSI in August 2004 for the larger and smaller size classes was 12.8% and 11.4% respectively. In December (summer) 2004 the gonads of the larger size class were completely spent with no discernable difference between the male and female sections of the gonads. The mean GSI for this period was only 5.2%. The GSI in both size classes started to increase again in January 2005. The trend in gonad development indicates that *P. sulcicostatus* might reach its spawning peak in winter. The mean concentrations of chlorophyll *a* in winter are lower during spring, summer and autumn. Wind also shows a strong seasonality over False Bay, with strong south-easterly winds dominating in summer, resulting in localized upwelling. Because of a significant stratification, the bottom temperature is 1°C to 3°C lower in summer than in winter.

**RISE AND FALL OF THE CALICO SCALLOP (*ARGOPECTEN GIBBUS*) FISHERY OF FLORIDA, USA.** Bill Arnold. Fish and Wildlife Research Institute, Florida Fish and Wildlife Conservation Commission, 100 Eighth Avenue SE, St. Petersburg, Florida 33701, USA.

The calico scallop (*Argopecten gibbus*) supports an important commercial fishery on the east coast of Florida, USA. Peak landings were realized from the east Florida shelf during the 1980s,



with substantially fewer landings before and after that time. That pattern of landings could indicate that scallops were unusually abundant during the 1980s and that pre- and post-1980s landings data reflect more typical standing stock values. However, results from exploratory fishing cruises conducted by federal fishery research vessels beginning in the late 1940s and continuing through the 1960s suggest that large stocks of calico scallops were extant off North Carolina, east Florida, and in the Gulf of Mexico. Those stocks were not exploited until the 1980s because the processing machinery necessary to efficiently handle the landed product was not previously available. Once an efficient processing method was developed and implemented, the fishery rapidly expanded and calico scallop populations on the east Florida shelf were intensively exploited. Subsequent to the 1980s, harvest and processing capabilities remained in place, but commercial landings fell to near zero. It is therefore possible to define three phases in the history of the calico scallop fishery in Florida. During phase 1 (approximately 1950–1980), exploratory fishing activities indicated that large numbers of scallops were known to exist on the east Florida shelf and in other areas of Florida. Those exploratory fishing efforts extended over many years, indicating that the abundance of calico scallops was not an ephemeral situation but was instead a relatively constant feature of the east Florida shelf. During phase 2 (approximately 1980–1990), the needed processing equipment was deployed and the commercial fishery expanded rapidly. To estimate harvest during this phase of the scallop fishery, consider that a large calico scallop adductor muscle weighs approximately 4 g, equivalent to about 110 scallops harvested per pound of meat landed. Thus, during 1984 alone, more than 4.4 billion scallops were harvested. During phase 3 (approximately 1990 to present), the scallop population on the east Florida shelf declined and the commercial industry went with it. Small numbers of scallops were harvested from that area during the early 1990s, but the commercial fishery at the beginning of the 21st century is essentially non-existent. Possible causes of the purported collapse include one or a combination of the following factors: overfishing, habitat loss, disease, or natural variation. Fishing activities target high-density patches of scallops, so it is possible that removal of large numbers of scallops during the 1980s eliminated many high-density patches and reduced the reproductive viability of the population, ultimately leading to population collapse. Harvesting activities also remove large amounts of calico scallop and other shell. That shell provides a primary settlement substrate for calico scallops in what is an otherwise featureless sand bottom. Thus, fishing activity has resulted in the destruction of the very habitat upon which recruiting scallops depend. A protistan parasite of the genus *Marteillia* was first recorded from the east Florida calico scallop beds in 1991. Although there is no direct evidence that this parasite caused mortality in the scallop population, species of *Marteillia* have been implicated in other bivalve mass mortality events. Finally, the east Florida shelf is characterized by a dynamic oceanographic regime that is strongly influenced by Gulf Stream meanders

and resultant seasonal upwelling. Changes in that regime, especially relatively long-term changes associated with ENSO or NAO events, could have altered the population dynamics of calico scallops on the east Florida shelf. Economic factors may prevent the return of the calico scallop fishery in Florida even if the scallop population rebounds. First, a bay scallop (*Argopecten irradians*) aquaculture industry has been developed in China, and that industry produces scallop meats that can be shipped to the United States and sold more cheaply than calico scallops can be harvested from the wild fishery and sold in the United States. Thus, there is little economic incentive to invest in the calico scallop industry even if the animal once again became abundant. Second, Port Canaveral, Florida is the primary landing port for calico scallops on the east coast of Florida but also has become a major cruise ship terminal. Cruise ship activities and calico scallop processing activities are somewhat incompatible because of the odor and noise that emanate from the processing facilities. Additionally, shoreside sites that once supported processing facilities are now being converted to more lucrative marinas and terminals, with the result that there is simply no room for the calico scallop industry at Port Canaveral.

**A BRIEF HISTORY OF BAY SCALLOP (*ARGOPECTEN IRRADIANS*) RESTORATION EFFORTS IN FLORIDA USA WATERS.** Bill Arnold<sup>1</sup> and Norman J. Blake<sup>2</sup>. <sup>1</sup>Fish and Wildlife Research Institute, Florida Fish and Wildlife Conservation Commission, 100 Eighth Avenue SE, St. Petersburg, Florida 33701, USA; <sup>2</sup>College of Marine Science, University of South Florida, 140 Seventh Avenue S, St. Petersburg, Florida 33701, USA.

Bay scallops (*Argopecten irradians*) once supported important commercial and recreational fisheries in Florida waters, and archaeological evidence suggests that they contributed to the cultural fabric of native Floridians many centuries prior to European invasion. However, in recent decades many of the local populations that comprise the Florida metapopulation of bay scallops have collapsed with the result that the entire metapopulation may be imperiled. In an effort to stabilize the bay scallop metapopulation in Florida, the State of Florida first instituted severe harvest restrictions and then instituted a restoration program. The goals of this combined passive-active approach to rebuilding scallop populations were to (1) prevent further loss of reproductively viable populations and (2) rebuild populations in areas where habitat appeared to be amenable to supporting viable scallop populations. Historic and anecdotal information indicates that bay-scallop populations in Florida waters once extended from at least West Palm Beach on the east coast to Pensacola in the Florida panhandle. By the early 1980s, all populations from Anclote south had essentially collapsed. At that time, the State instituted regulations to create both a harvest season and a five-gallon (19 L) bag limit. By the early 1990s, additional populations between Anclote and

Crystal River also appeared to have collapsed. In response, the State implemented additional harvest restrictions and initiated a population restoration program that continues to this day. The success of those management and restoration efforts is equivocal and is the topic of this presentation. Modifications to the bay scallop management regime were initiated in 1994 with the complete closure of the commercial fishery, closure of the recreational fishery south of the Suwannee River, reduction of the fishing season from 9 months to 3 months, and a reduction in the bag limit to two gallons (7.6 L) per person per day. The next year, the season was further reduced to approximately 2 months per year. However, despite these drastic restrictions on harvest, no substantial increase in scallop abundance was observed between the years 1994 and 1998. Efforts to restore local scallop populations were instituted in 1998 as a joint effort between the Florida Fish and Wildlife Conservation Commission and the University of South Florida College of Marine Science. This effort involved planting juvenile scallops at high density in protective cages at various sites along the SW coast of Florida. Those juveniles would then grow to adulthood within the cage, thus ensuring high density and relatively high fertilization efficiency during subsequent spawns. The resultant offspring would then settle to the local seagrass beds and thereby repopulate the area. Population monitoring within the target area indicated that scallop density did increase according to prediction in at least a subset of the target area. Scallops were first planted in spring 1998, subsequently achieved adulthood, and spawned during fall 1998. Thus, it would be expected that offspring from those planted scallops would achieve adulthood in spring 1999 and would be detected during sampling surveys conducted in the summer of 1999. That expectation was realized as an order-of-magnitude increase in scallop abundance at the Homosassa study site between June 1998 and June 1999. Additional planting efforts were conducted in 1999 and 2000, with the result that scallops increased by yet another order of magnitude between June 1999 and June 2000 and remained similarly abundant in 2001. However, funding for the restoration effort ended in 2000 so no scallops were planted in spring 2001. Scallop abundance immediately decreased in the Homosassa target area, and by June 2004 scallop abundance had fallen to background levels despite a continuing but smaller-scale restoration effort. Initial interpretation of the results of management and restoration activities, including data acquired through summer 2001, suggested that efforts to rebuild scallop populations in Florida waters had been successful although it was not clear if management, restoration or a combination of the two was responsible for the resurgence. Inclusion of more recent data, as described earlier, suggests that restoration activities may have been the pre-eminent factor, because scallop abundance at the Homosassa restoration site rose and fell in concert with restoration activities. Whether governmental organizations are willing to devote the necessary resources to a continuing scallop restoration program remains to be seen.

**SEASONAL VARIATIONS IN CHEMICAL, PHYSICAL, TEXTURAL AND MICROSTRUCTURAL CHARACTERISTICS OF ADDUCTOR MUSCLES OF GIANT LION'S PAW SCALLOP (*NODIPECTEN SUBNODOSUS*).** Ana I. Beltrán-Lugo,<sup>1</sup> Alfonso N. Maeda-Martínez<sup>1</sup> and Ramón Pacheco-Aguilar.<sup>2</sup> <sup>1</sup>Centro de Investigaciones Biológicas del Noroeste (CIBNOR), Mar Bermejo No.195, Col. Playa Palo de Santa Rita, La Paz, Baja California Sur 23090, México; <sup>2</sup>Centro de Investigación en Alimentación y Desarrollo (CIAD), Carretera a la Victoria Km. 0.6 Hermosillo, Sonora 83000, México

The giant lion's paw scallop (*Nodipecten subnodosus*) is one of the most commercially important species of scallops in Mexico. The quality of its adductor muscle, the main edible portion, depends greatly on texture and color characteristics. The texture of fish muscle is determined by many factors, including chemical composition (Crapo et al. 1999) and muscle structure (Bjørnevik et al. 2004). These characteristics vary seasonally in response to reproductive activity and availability of food. For lion's paw scallop, how the quality parameter is influenced by seasonal variations in the composition of the adductor muscle have not been investigated. This study analyzed the effects of season on chemical, physical, textural and microstructural characteristics of adductor muscles of giant lion's paw scallop (Fig. 1). Samples of scallops (average size  $66.3 \pm 6.9$  mm) were harvested in winter (January), spring (April), summer (September) and fall (November) 2003 from an aquaculture farm on the Pacific coast of the Baja California Peninsula, México. Morphometric variables were recorded for a sample of 30 specimens to calculate muscle index and gonadosomatic index. These indices were used as indicators of physiological condition of the organisms at the time of harvest. To assess the quality of the adductor muscles; proximate composition (moisture, protein, lipids, ash, and carbohydrates); postmortem pH; color parameters ( $L^*$ ,  $a^*$  and  $b^*$ ); water-holding capacity; texture (Warner-Bratzler shear test) and microstructure were analyzed (Fig. 2). We observed that organisms sampled in summer showed higher muscle and gonadosomatic indices. Chemical composition varied throughout the year. Levels of moisture and lipids were significantly lower in the summer samples but carbohydrate content was higher. Post-mortem pH in summer was lower than other seasons. Lower water-holding capacity in the summer samples seems to be influenced by the lower pH in adductor muscles. Slight but significant changes in color characteristics of adductor muscles were observed in all seasons. Color is known to be related to lipids content; however, we found no support for seasonal color differences to be related to this chemical component. Warner-Bratzler shear test values yielded the poorest muscle texture in summer; most likely related to lower pH, water-holding capacity, and wider spacing between muscular fibers and structural alterations at this season. Our results indicate that the quality of adductor muscle is somewhat poorer if collected in summer even though scallops yield the highest muscle weight of all seasons. In contrast, the quality of muscles in winter is the highest, but the muscle yield is 11.5% lower than the summer.

With these results and the parameters of quality that the markets demand, the scallop farmer will be able to refine its harvest program.

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**CLOSED AREAS AND STOCK ENHANCEMENT OF SCALLOPS—WHAT'S THE CATCH?** Bryce D. Beukers-Stewart, Belinda J. Vause, Matthew W. J. Mosley and Andrew R. Brand. Port Erin Marine Laboratory, University of Liverpool, Port Erin, Isle of Man IM9 6JA, British Isles.

The use of closed areas and stock enhancement to manage fisheries is becoming increasingly popular around the world. Scallop fisheries appear to be particularly suited to this type of management, which has produced highly productive and stable fisheries in countries such as Japan, China, New Zealand and the United States. A small area (2 km<sup>2</sup>) off the SW coast off the Isle of Man, British Isles, has been closed to fishing by towed gear (i.e., dredges and trawls) since 1989. During closure the density and biomass of great scallops (*Pecten maximus*) in the closed area has increased at an accelerating rate such that by 2004 the density, exploitable biomass and reproductive biomass of scallops was 5.5, 8.0 and 9.5 times greater, respectively, than on the adjacent fishing ground. In addition, scallop recruitment and commercial catch rates have also increased during the study period on the several fishing grounds surrounding the closed area. These patterns may be the result of very high levels of scallop reproduction in the closed area and subsequent larval export, and/or they could be explained by spillover (movement) of scallops from the closed to the fished areas. However, fluctuations in scallop populations can also be driven by various other factors such variation in hydrographic conditions or predator abundance. Identifying and quantifying the mechanisms by which closed areas may benefit fisheries is crucial if they are to be adopted as a widespread management tool. This study describes a new project examining the effectiveness of combining closed area management with stock enhancement. The initial phase of this project involved importing juvenile scallops from the Isle of Skye, Scotland for direct seeding on the seabed. A small area of seabed (~1 km<sup>2</sup>), adjacent to the original closed area, was closed to mobile fishing gear for 3 years from June 2003 to protect these scallops while they grew to commercial size. It was envisaged that this reseeded exercise would “kick start” the recovery of the newly closed area. Protecting the scallops during their critical juvenile phase should also improve their survival and growth – enhancing yields when the area is re-opened to fishing. In addition, because the reseeded scallops were likely to be genetically different from

the local stock, it was intended to use this feature to track their dispersal and potentially quantify any larval export from the new closed area. In an attempt to reduce the number of predators (starfish and crabs) prior to re-seeding, the area to be closed was initially subject to 2 days of commercial scallop dredging at the end of May 2003. It was calculated that the entire seabed within this area was dredged at least twice during this procedure. Dive surveys were then done to obtain background estimates of the density of scallops and other benthic species. For the reseeded exercise, approximately 45,000 scallops that had been reared in suspended culture were collected from the Isle of Skye in mid June 2003 and transported to the Isle of Man using a vivier lorry. Scallops came from two different farms and were mainly 2- (42%) and 3- (53%) year-old individuals. Mean sizes were 55.4 mm SL for 2-year olds and 69.2 mm SL for 3-year olds. Scallops from one farm were very heavily encrusted with epifauna, mainly barnacles, and both sets of reseeded scallops were easily distinguished from local scallops by their size and appearance. On arrival at the Isle of Man the reseeded scallops were transported by boat to a designated 50 m × 100 m plot within the new closed area and released. Survival after 2 weeks was assessed in the laboratory and the field via diver surveys. The encrusted scallops suffered substantial mortality in the laboratory (>80%), whereas the clean batch showed greater than 95% survival. In the field the respective figures were 33% survival for the encrusted scallops and 60% for clean scallops (total mean survival 42.3%). These diver surveys also revealed a dramatic aggregation of predators (mainly the common starfish *Asterias rubens* and edible crab *Cancer pagurus*) in the reseeded plot, whose densities had increased 20-fold since the background survey. Thirty percent of dead 2-year-old scallop shells displayed obvious crab damage, compared to only 12% of dead 3-year-old scallop shells. Dive surveys were repeated in September 2003 and July 2004 (3 and 12 months after reseeded respectively), both within the reseeded plot and at various distances outside up to 500 metres away. After 3 months the density of live reseeded scallops in the plot had dropped dramatically to less than one percent of the original reseeded density. However, there was no corresponding increase in the density of dead scallops; in fact this had dropped by 75%. In comparison, the density of scallops outside the plot had increased from 0.75 m<sup>-2</sup> prior to reseeded to 5.0 m<sup>-2</sup> in September 2003, and remained high in July 2004 (3.9 m<sup>-2</sup>). These patterns seem to have been strongly driven by dispersal of scallops from the reseeded plot, because there was very little natural recruitment during this period. Reseeded scallops were found up to 500 m from the reseeded plot and accounted for at least 28% of all scallops in the new closed area in July 2004. Based on these estimates it was calculated that at least 10,920 reseeded scallops were alive in July 2004. These scallops all appeared to belong to the clean batch, giving a survival rate of 52% for these scallops but no survival of the encrusted type. Preliminary work has also demonstrated that it is possible to distinguish between the Skye and Manx scallops

using microsatellite analysis. Samples collected during the dive surveys and from spat collectors in 2004 are currently being analyzed to refine our estimates of scallop dispersal and investigate the possibility of larval export from breeding among the reseeded scallops. Future reseeding exercises would benefit from ensuring that the scallops to be reseeded are in good health and that the area to be reseeded is more effectively cleared of predators. However, the reseeding exercise does appear to have kick-started the recovery of scallop populations in the new closed area. This should lead to increased breeding within this area and potentially larval export to the surrounding fishing grounds. The genetic distinctness of the Skye scallops may allow larval export to be quantified. An experimental fish-down of the new closed area is planned for mid 2006 to assess the fisheries benefits of enhancing and closing an area for 3 years. Given our results to date, a combination of closed area management and stock enhancement seems to hold considerable promise for improving the inshore scallop fishery around the Isle of Man.

**GENOTYPIC EFFECTS OF GLYCOLYTIC ENZYMES ON THE LEVELS OF ENERGY SUBSTRATES IN GONAD AND MUSCLE OF *ARGOPECTEN PURPURATUS*.** Katharina Bröckordt and Federico Winkler. Center for Advanced Studies in Arid Zones (CEAZA) and Marine Sciences Faculty, Universidad Católica del Norte, Coquimbo, Chile.

The polygenic nature of most quantitative traits makes difficult to understand the biochemical genetic basis of phenotypic variation. One way to examine the interactions between the forces that outline the phenotype of an organism with those that maintain its underlying genetic variation, is to inspect the variation at a specific gene, especially one whose biochemical function is known, and

then recognize its effect on the phenotype (Krause & Bricelj 1995). Glycolytic and pre-glycolytic enzymes have an important function in the storage and utilization of energy substrates in the gonad and the mussel. We examined the genotypic effect of 5 glycolytic and pre-glycolytic polymorphic allozymes (ODH, APK, GPI, G3PDH and PGD) on the variation in energy substrate contents (carbohydrates, lipids and proteins) present in gonad and adductor muscle of adult allozymes genotype was assessed in 180 scallop muscles by horizontal starch gel electrophoresis. In the same individuals the content of carbohydrates, proteins and lipids in the muscle and gonad were determined using the methods described by Dubois et al. (1956), Lowry (1951) and Mann and Gallager (1985), respectively. *A. purpuratus* muscle showed a significant variation on carbohydrate content, related to the genotype of *ODH\**, *APK\** and *G3PDH\** (Fig. 1). The presence of the common alleles *ODH*<sup>110</sup> (homozygote), *APK*<sup>142</sup> and *G3PDH*<sup>85</sup> was related to the highest levels of muscle carbohydrates. Muscle protein content was highest when the alleles *G3PDH*<sup>85</sup>, *PGD*<sup>108</sup> and *PGD*<sup>80</sup> were present. Lipid content in the muscle was not affected by any allozyme genotype we tested. In the gonad, the occurrence of the alleles *ODH*<sup>110</sup> and *APK*<sup>142</sup> was related to the highest levels of carbohydrates and lipids. In addition, gonad carbohydrate content was greater when the allele *GPI*<sup>108</sup> was present (Fig.2). Finally, the occurrence of the alleles *APK*<sup>142</sup>, *G3PDH*<sup>85</sup>, *GPI*<sup>108</sup> and *PGD*<sup>80</sup> implied a higher level of proteins in the gonad. Our results show a clear association between the *ODH\**, *APK\**, *GPI\**, *G3PDH\** and *PGD\** genotypes and the levels of the three energetic substrates in the adductor muscle and gonad of *A. purpuratus*. Considering the great importance of these tissues in reproduction and escape response, the occurrence of alleles associated with highest contents of carbohydrates, lipids or proteins may have an adaptive importance to *A. purpuratus*. The effect of specific alleles on productive

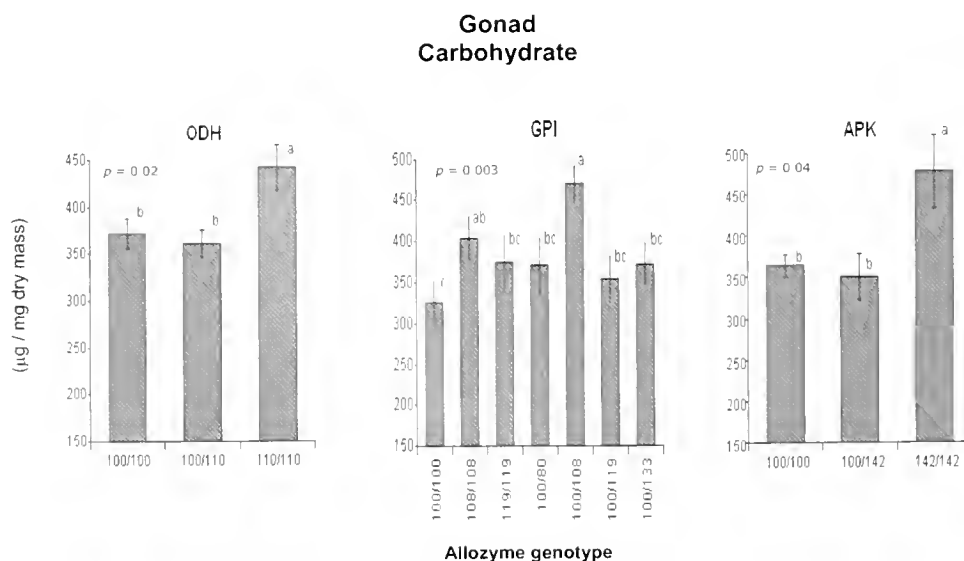
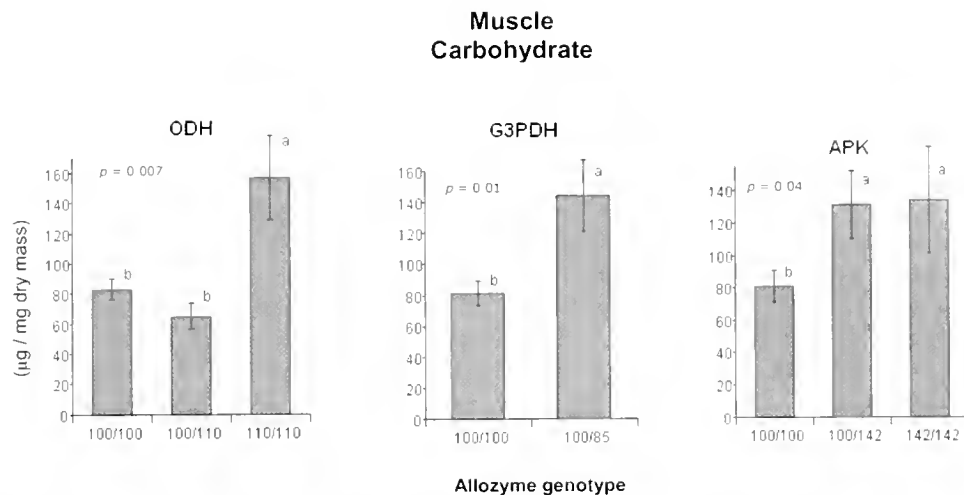


Figure 1. *Argopecten purpuratus*. Effect of genotype at *ODH\**, *G3PDH\** and *APK\** loci on muscle carbohydrate content.



**Figure 2.** *Argopecten purpuratus*. Effect of genotype at *ODH*\*, *GPI*\* and *APK*\* loci on gonad carbohydrate content.

traits also should be considered for genetic management strategies in scallop aquaculture.

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**EMBRIOLOGICAL DEVELOPMENT OF CATARINA SCALLOP *ARGOPECTEN VENTRICOSUS*.** Carlos Cáceres-Martínez,<sup>1</sup> Jorge Chávez-Villalba,<sup>2</sup> Jorge I. Cáceres-Puig<sup>1</sup> and Jorge A. Massó-Silva.<sup>1</sup> <sup>1</sup>Universidad Autónoma de Baja California Sur, Km 5.5 Carretera al Sur, La Paz, BCS, 3080, México; <sup>2</sup>Centro de Investigaciones Biológicas del Noroeste, Unidad Guaymas, AP 349, Guaymas, Sonora 85465, México.

During the last 25 years different efforts have been made for the knowledge of biological history of *Argopecten ventricosus* in the North West of Mexico, in spite of the capability of hatchery seed production and the knowledge of larvae development, the embryology of this species remains undescribed. The objective of this work is to describe the morphological changes during fecundation, segmentation and gastrulation of *A. ventricosus*.

Twenty-five scallops were collected in La Paz Bay and transported to the laboratory. After they were cleaned, they were stimulated by thermal shock for spawning. When spawning was initiated, the scallops were set in individual containers to obtain separated gametes, male and female gametes were combined and the embryological development was followed until the appearance of the trocophore larvae. The incubation condition took place in filtered (1 µm) and UV irradiated seawater maintained at 21 °C in three 10-L plastic containers. After fertilization, samples were taken for microscopic, micro-photographic and micro-video ob-

servations. All the changes were recorded for description of embryonic development. The eggs of *A. ventricosus* were spherical with diameter of 45 µm average, which means, quite small in comparison to *Pecten maximus* eggs (79 µm) (Paulet et al. 1988). After 29 minutes from fertilization, we observed the first polar body on 15% of the eggs, in vegetal pole 15 minutes later we observed the first division where the first segmentation plan (meridional) was easily recognized. The segmentations in scallops were described for *Chlamys opercularis*, *Placopecten magallanicus*, *Argopecten irradians*, *Patinopecten yessoensis*, *Chlamys hastate* and *Pecten maximus* as was noticed by Casse (1995). During the first division, we observed the formation of the polar lobule and its fusion with the blastomere. One hour and 25 minutes after fertilization, the second segmentation edge appeared, the second division was present in the 10% of embryos, 1 hour 45 minutes after fertilization and 5 minutes later, 80% of embryos showed the third division (equatorial). After the fourth division, we could not differentiate the segmentation plans until they reached the stereoblastule stage, ending the segmentation process and beginning the gastrulation with morphogenic movements as epibolia and invagination for the formation of blastopore in the vegetative pole. As it was reported for molluscs, the gastrula stage was ciliated (Cragg, 1980), and its movement maintained the embryo rotating over by itself all the time. The experiment finished 6 hours 12 minutes after the fertilization started, when the trocophore larvae were functional.

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**ROUND SCALLOPS AND SQUARE MESHES—PROMISING FIELD TRIALS WITH BYCATCH REDUCTION DEVICES (BRDS) REDUCE BOTH BYCATCH AND UNDERSIZED SCALLOP CATCHES.** Matthew Campbell,<sup>2</sup> Keith Chilcott,<sup>2</sup> Anthony Courtney,<sup>2</sup> Shane Gaddes,<sup>2</sup> Peter Kyne,<sup>1</sup> Darren Roy,<sup>2</sup> Mark Tonks<sup>2</sup> and Michael O'Neill.<sup>2</sup>

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Benthic trawl fisheries, such as those for prawns and scallops on the Queensland east coast, generally produce large amounts of bycatch, which is comprised of hundreds of species of small fish and invertebrates. The amount of bycatch produced greatly exceeds that of the target catch. A research project funded by the Australian Fisheries Research and Development Corporation (FRDC) quantified the catch rates and composition of bycatch in the fishery and tested a range of bycatch reduction devices (BRDs). A 9-day research charter conducted in the scallop fishery demonstrated that bycatch could be reduced by 78% by using nets with a turtle excluder device (TED) and a square mesh codend BRD installed, compared to a standard net. If scallop fishers used the devices, it would reduce bycatch by several thousand tons annually. Whereas the research was undertaken to address bycatch, it also found that the devices reduced catch rates of undersized scallops by 63%. Square mesh codends, in particular, show great potential for regulating the minimum size of scallops and lowering incidental fishing mortality, as well as significantly reducing bycatch.

**A STEREOLOGICAL STUDY OF THE REPRODUCTIVE CYCLE OF *PECTEN MAXIMUS* IN MÁLAGA. EFFECTS ON ITS AQUACULTURE.** Jesús Campos,<sup>1</sup> Juana Cano,<sup>2</sup> Carmen Vázquez<sup>1</sup> and Francisco J. López.<sup>2</sup> <sup>1</sup>Centro Oceanográfico de A Coruña, Instituto Español de Oceanografía, PO Box 130, 15080 A Coruña, Spain; <sup>2</sup>Centro Oceanográfico de Málaga, Instituto Español de Oceanografía, PO Box 285, 29640 Fuengirola (Málaga), Spain.

The great scallop, *Pecten maximus* L., is a bivalve belonging to the family Pectinidae. This species is distributed all along the east coast of the Atlantic Ocean, from Norway to Mauritania, and enters the Alboran Sea. Numerous studies have been carried out on the reproductive cycle of this species at different locations on the European coast, pointing to the varying behavior of the great scallop depending on the zone and year when there are different environmental conditions. (Paulet et al. 1988, Paulet & Boucher 1991, Strand & Nylund 1991, Pazos et al. 1996). To develop culture systems of a particular species, it is necessary to have an understanding of its reproductive biology. In 1997 studies began with a view to develop the culture of the great scallop in Málaga, which has the largest extension of natural banks on the Iberian

peninsula. Up until the year 2001 the reproductive cycle had only been studied in wild specimens of this species collected from the naturally occurring populations near the area where the culture is carried out (Román et al. 2001). Cano and Campos (2003) studied the reproductive cycle, age at onset of maturity and spawning season of cultured scallops on the basis of gonad condition indices, and they compared these data with the reproductive cycle of wild scallops, finding the sexual behavior of both scallops to be similar. This work discusses the knowledge of the reproductive biology of *P. maximus* on the basis of the evolution of the gonad condition indices as well as using stereological studies and oocyte size frequency, which are the most objective methods available to evaluate sexual maturity. The culture was carried out in a park located opposite the fishing port of Fuengirola (Málaga), which has mean depths of 20 m. Spat was obtained from collectors moored from April to July, 2001 and removed in September and October. Spat detached in September and October were moored on a longline at 10-m depth below the bottom in round culture trays 40 cm in diameter at a stocking density of 40 scallops/tray. In January, 2002 the animals were thinned out to a stocking density of 24 scallops/tray and in June, to 16 scallops/tray. Starting in January, 2002, 30 scallops were sampled monthly in 2002 and at intervals of every 2 weeks in the 2003. Scallop shell length and height (mm) were measured and the whole animal was weighed (g). The scallops were then dissected separating the muscle, gonad, digestive gland and remaining tissues (gills + mantle + foot), and wet weight as well as shell dry weight (g) were obtained. These values were used to calculate the condition indices of the different organs:  $CI_{organ} = 100 * (\text{Shell weight} / \text{Organ dry weight})$ . The gonads of 10 specimens were processed using traditional histological methods. Ten images of each gonad were randomly selected for analysis using stereological methods (Paulet 1990, Mestre 1992, Pazos et al. 1996). The mean diameter and area of each oocyte were recorded by counting and automatic measuring, and objects were selected by color range and size. Each gonad yielded data for more than 300 oocytes. The gonad occupancy index was also calculated ( $GOI = \% \text{ occupied by oocytes that had not undergone atresia}$ ) for each image, taking into account the area occupied by the oocytes and the size of the standard visual field ( $143,939 \mu m^2$ ). In 2002 the gonad condition index of the cultured scallops rose from January until May (1.07), followed by a drop until the month of September at which time recovery began with a second maximum being reached in January 2003 (2.22). In 2003 the index was seen to peak and drop consecutively from January to May (2.79). After this, it increased again in August (1.36) and dropped down to a minimum value of 0.5 in October. The summer rise was not observed in 2002, however we must bear in mind that the samplings were carried out monthly during that year. In fact, in a simultaneous experiment performed on wild scallops where sampling was done at intervals of every 2 weeks, values increased in mid-April and again at the beginning of June. These values would not have been recorded if sampling had been carried out monthly. The stereo-

logical analysis of the gonads shows that the volume of occupancy went from 0.5% in September 2002 to 95% in August 2003. Mean oocyte diameter ranged from 7.30  $\mu\text{m}$ , corresponding to the few oocytes found in some of the gonads in September 2002, to 40.07  $\mu\text{m}$  in August of the same year. The gonads were found to be lacking intrafollicular space, but had a considerable proportion of lysis. Oocyte atresia or lysis presented maximum values in late May, July and August, 2002 and in April, 2003, which was associated with an abundant proliferative activity and this might be seen as a response to the liberation of intrafollicular space, as happens during partial spawnings (C. A. Borzone et al. 2003). The greatest proportion of intrafollicular space would coincide with the spawnings recorded at the end of June and in September 2002 (total emptying of the gonad) and in early April, in July and late August in 2003. Because considerable lysis was found in August and to a lesser extent in May of 2002 and April 2003, it was assumed that these spawnings were not the best ones to be used in the procurement of spat. Cano and Campos (2003), reported that, in effect, the highest number of new attachments of this species took place in the collectors in April 2002 and in June, July and August of 2003. The size frequency distribution of the oocytes is in keeping with the gametogenic cycle described previously despite the fact that in most cases, the distributions showed that the follicles contain oocytes in varying stages of development. In 2002, we observed an increase in the frequencies of the larger sizes without the incorporation of new cohorts of young oocytes in May, which is characteristic of maturation, whereas in October, only oocytes measuring less than 35  $\mu\text{m}$  were found to be present, indicating that one spawning had taken place. In 2003, there was an increase in the larger sizes in March, May and early August with the incorporation of young oocytes primarily in April, June and September. Based on all these data the reproductive cycle of the great scallop in Málaga was concluded to be characterized by consecutive spawnings and recoveries from March until late summer, and the incidence of oocyte atresia depends on environmental conditions, basically temperature and the availability of food, with the spawnings producing greater or lesser spat settlement, accordingly.

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**REPRODUCTIVE CYCLE OF *MIMACHLAMYS VARIA* IN MÁLAGA AND EFFECTS ON ITS AQUACULTURE.** Jesús Campos,<sup>1</sup> Juana Cano,<sup>2</sup> Carmen Vázquez<sup>1</sup> and Francisco J. López.<sup>2</sup> <sup>1</sup>Centro Oceanográfico de A Coruña, Instituto Español de Oceanografía, PO Box 130, 15080 A Coruña, Spain; <sup>2</sup>Centro Oceanográfico de Málaga, Instituto Español de Oceanografía, PO Box 285, 29640 Fuengirola (Málaga), Spain.

The settlement of *Mimachlamys varia* in Málaga is very high and preliminary data on the tray culture of this species would suggest that it is a good candidate for aquaculture in this area (Campos & Cano 2003). The reproductive cycle of this scallop, which is essential in establishing the relationship between spawning and recruitment to develop the culture, has been studied under Project ACU00-008-C3-1 financed by the INIA. This research entailed a study of the gonad condition index as well as a histological study of the gonad, by means of stereological methods that shed light on gonad evolution and oocyte atresia. Spat removed from the collectors moored in 2001 were used. Over the course of 2 years, 2002 and 2003, 30 specimens were sampled monthly, and the scallops were separated by sex (males and females). Shell length and height (mm) were measured and the whole animal was weighed (g). The scallops were then dissected, separating the muscle, gonad, digestive gland and other tissues (gills + mantle + foot), and wet weight as well as shell dry weight were recorded (g). These were used to calculate the condition indices of the different organs:  $CI_{organ} = 100 * (\text{shell weight} / \text{organ dry weight})$ . In addition to the analysis of the female gonads, quantitative analyzes were performed using stereological methods (Paulet 1990, Mestre 1992, Pazos et al. 1996); 10 specimens were processed according to traditional histological methods. Ten images of each gonad were randomly selected for analysis. The mean diameter and area of



each oocyte were recorded by counting and automatic measuring, and objects were selected by color range and size. In general each gonad yielded data for more than 300 oocytes. The gonad occupancy index was also calculated ( $GOI = \% \text{ occupied by oocytes that had not undergone atresia}$ ) for each image, taking into account the area occupied by the oocytes and the size of the standard visual field ( $143,939 \mu\text{m}^2$ ). During the 2 years of the study, the male and female gonad condition indices present a similar cycle, maximum values occurring from February to May, after which they decline until they reach minimum values in August to September with recovery and maturation beginning again in winter. During the second year, values peaked in February followed by a sharp drop and quick recovery in March. With the onset of maturity, most of the gonads are male, but the relation between males and females changes over the course of the reproductive cycle so that the proportion of females is greater—as high as 70%—during the maturation stages, dropping to as low as 20% afterwards. The stereological analysis shows that from February to August partial spawning takes place and scallops recover from these events quickly. During these stages mature oocytes and pre-vitellogenic oocytes are visible on the follicle walls. In 2002, the greatest occupancy was seen in May and June (91.34%, 90.67%) and the gonads were practically empty in August and October (1.7% y 2% respectively). Maximum GOI occurred in February 2003 (95.90%) with recovery taking place from April to June. The mean diameter of the oocytes ranged from 18.35–35.18  $\mu\text{m}$ . However, this is an underestimation because a gonad contains oocytes of varying sizes. The size frequency distribution of the oocytes indicates that a proliferative activity takes place practically throughout the whole year. The only time oocytes of  $<10 \mu\text{m}$  do not appear is during the months of complete maturation in spring and early autumn, when the gonad is almost completely empty. Oocyte atresia or lysis was found in most samples, however it was highly abundant in the terminal stages of vitellogenesis in winter and summer. Therefore, we can see that although on February 5, 2003 occupancy amounted to 95.96% with the absence of lysis, on 21 February lysis was already 31%, and the high occupancy seen on 20 June (98%) was followed by a lysis of 44.5 % on 15 July. This may have been because of the fact that the conditions were not optimum for spawning and mature oocytes have a relatively short period of viability in the follicles. The spawning period is broad-ranging and lasts from February until late summer, but when there are high levels of oocyte atresia during the reproductive cycle, this leads to substantially low values on the gonad condition index, which are an impediment to the abundant settlement of spat in the collectors. This occurrence is supported by settlement data from the collectors moored to study the recruitment pattern of this species, where we observed that the highest settlements recorded over the 2-year study pertained to the collectors moored in April and removed in June and July. This corresponds to the release of gametes after the maximum maturation stages in April and May (Cano & Campos 2003). This is the time of year that the collectors should be moored

for the procurement of seed from the natural environment to be used in culture.

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**THE INFLUENCE OF DENSITY AND DEPTH ON THE GROWTH AND SURVIVAL OF *MIMACHLAMYS VARIA* AND *AEQUIPECTEN OPERCULARIS* IN MÁLAGA (SPAIN).** Jnana Cano,<sup>1</sup> Jesús Campos<sup>2</sup> and Francisco J. López.<sup>1</sup> <sup>1</sup>Centro Oceanográfico de Málaga, Instituto Español de Oceanografía, PO Box 285, 29640 Fuengirola (Málaga), Spain; <sup>2</sup>Centro Oceanográfico de A Coruña, Instituto Español de Oceanografía, PO Box 130, 15080 A Coruña, Spain.

For the development and optimization of a species in suspended culture it is well known that one of the most important aspects is to determine the depth and stocking density at which the species will thrive, resulting in the highest growth and lowest mortality rates. Málaga is an area of interest for the development of pectinid culture in Spain. The three commercial species (*Pecten maximus*, *Mimachlamys varia* and *Aequipecten opercularis*) have a high settlement rate, with an average of around 200 specimens per collector for the three species (Cano et al. 1999, Cano et al. 2003). Studies have been carried out to determine the optimum density-depth for the culture of *P. maximus* (Cano et al. 2000), but not for *M. varia* and *A. opercularis*. The study of these two species began in 2002 within the framework of Project ACU00-008-C3-1 financed by the INIA. Scallops were reared in Fuengirola (Málaga). Spat were procured from collectors moored between April and June and removed in October 2002. The suspended culture of the scallop (*M. Varia*) was started on October 24, 2002 and that of the queen scallop (*A. opercularis*) began on November 4, 2002. The aim of the experiment is to study the optimum density and depth to be used in culture. Trays containing *Mimachlamys varia* were moored on the longline at 5- and 15-m depths below the bottom. Two initial sizes were used—average sized specimens (height =  $15.2 \pm 2.16$ ) and large specimens (height =  $22.43 \pm$



1.39)—and three stocking densities: 60 and 100 scallops/tray for the average sizes and 40 scallops/tray for the large specimens. The trays containing *Aequipecten opercularis* were moored at 6- and 12-m depths below the bottom. An initial large size was used (height =  $31.90 \pm 3.51$ ) and two stocking densities: 60 and 40 queens/tray. The scallops moored at 6 m and a density of 40 was lost due to a storm. In the two cases, the first sampling was carried out after 3 months (January and February, 2003), and the others in June, September and November 2003, which marked the end of the experiment. The samplings provided data on the size (height in mm), survival (number of live specimens in each sampling) and shell and flesh weight (gonad, muscle, digestive gland and remaining tissues) (g). *Mimachlamys varia*. The average-sized scallops moored at a density of 100 scallops/tray and at 5-m depth exhibited a mortality of 92% in the month of September, which forced us to terminate the experiment and remove all the culture trays. From the start of the culture, the scallops of this size moored at 5 m showed a higher growth rate for all the parameters sampled, than the ones moored at 15 m. The latter specimens, however, did not suffer any mortalities until the month of November, having a rate of 8%. In these trays moored at a depth of 15 m, growth was seen to slow down from September to November, and the scallops decreased in flesh weight and fractions. The average sized *Mimachlamys varia* scallop moored at a lower density (60 scallops/tray) and at a depth of 5 m also presented a high mortality rate in September (86.67%), which forced us to terminate the culture experiment. Growth was similar in both cases during the sampling of both depths until the month of September. Organ growth was similar at the two depths, although it was slightly higher at 5 m depth. The large scallops reared at a density of 40 scallops/tray exhibited a similar growth in size at both depths, but differed in total weight and organ weight, because these values were higher at 5-m depth. At both depths, the digestive gland decreased in November, whereas the gonad increased. This occurred at all depths and densities owing to the onset of maturation, as seen in the study on the reproductive cycle. Queen scallops moored at a stocking density of 60 individuals/tray had an overall greater growth in size and total weight at both depths, than those stocked at 40 individuals/tray. The gonad, muscle, digestive gland and the remaining tissues behaved similarly. The animals grew quickly during the first winter, reaching the legal size (50 mm in height) in June for queens at a density of 60 and in September for those stocked at 40 individuals/tray. The gonad underwent a sharp rise in growth during the first 3 months, going from 0.21 g in November to 1.30 g in February. From this point on, the gonad weight and the gonad condition index began to decline. Muscle weight rose from 0.69 g to 3.82 g in 1 year, showing a continuous growth throughout the whole year, whereas the muscle condition index fluctuated. The weight of the digestive gland and the remaining tissues and their condition indices grew steadily throughout the whole year. In general mortality was high, particularly in September, probably because the high temperatures. Based on the results, the following

conclusions may be drawn: From the time the culture started, the *Mimachlamys varia* scallops moored at high densities (100 scallops/tray) and at 5-m depth had a higher growth in all the parameters sampled than those moored at 15 m, however the latter specimens did not show any mortality until November (8%). In the trays moored at 15 m, the animals exhibited a reduction in growth from September to November, and a drop in flesh weight. The *Mimachlamys varia* scallops moored at lower densities (40 scallops/tray) grew at a similar rate at both depths. In contrast, the total weight and organ weight were higher at 5-m depth. At both depths the digestive gland decreased in November whereas the gonad underwent an increase. Growth in *Mimachlamys varia* is greater at a density of 40 individuals/tray and at 5-m depth below the bottom. Growth in the queen scallops was greater at a density of 60 individuals/tray and at both depths. The growth of this species does not appear to be affected by depth, unlike in *Mimachlamys varia*.

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**THE USE OF DIFFERENT GROW-OUT METHODS IN THE CULTURE OF *PECTEN MAXIMUS*, *MIMACHLAMYS VARIA* AND *AEQUIPECTEN OPERCULARIS* IN MÁLAGA (SPAIN).** Juana Cano,<sup>1</sup> Jesús Campos<sup>2</sup> and Francisco J. López.<sup>1</sup> <sup>1</sup>Centro Oceanográfico de Málaga, Instituto Español de Oceanografía, PO Box 285, 29640 Fuengirola (Málaga), Spain; <sup>2</sup>Centro Oceanográfico de A Coruña, Instituto Español de Oceanografía, PO Box 130, 15080 A Coruña, Spain.

In 2001 studies were conducted on the maximum time pectinid spat should remain in the collectors with a view to examine the growth and survival of three scallop species (*Pecten maximus*, *Mimachlamys varia* and *Aequipecten opercularis*) (Campos & Cano 2003a and 2003b). Moreover, in 2002, when the spat were detached from the collectors, we found a large quantity of *Mimachlamys varia* seed <0.5 mm in size which would filter through the mesh of the quarters used to grow-out small spat and get lost. We decided to moor the spat in clean collector bags to prevent losses, because this scallop spat quickly resettles, using its byssus threads as an attachment base. With the results obtained from previous studies and within the framework of Project ACU00-008-C3-1 financed by the INIA, we decided to carry out this research work to determine the feasibility of other systems as an alternative to trays to be used in pectinid culture. Spat were procured from

collectors moored between January and July and removed in September 2003. This year we decided to moor the three species of scallops, *Pecten maximus*, *Mimachlamys varia* and *Aequipecten opercularis* removed from the collectors, in trays and bags similar to the collectors used to procure the spat, and carry out a comparison of the results. Monitoring of the spat began after removal and continued until December 2004, when the last sampling was taken. Another grow-out experiment was performed using *Mimachlamys varia* spat, taking advantage of its natural attachment mechanism by means of the byssus. Scallops were hung using a rope covered with a self-digesting net, similar to the kind used in mussel culture. During the sampling we recorded the size (height in mm) and survival (number of live specimens in each sampling). Great scallop spat were moored after being sorted into large ( $27.12 \pm 5.32$  mm) and small ( $17.20 \pm 5.32$  mm) sizes. Initial stocking densities in the trays consisted of 60 large and 200 small seed of great scallop/tray and in bags containing 100 large great scallops and 200 small great scallops/bag. *Mimachlamys varia* spat were separated into large ( $27.32 \pm 2.34$  mm) and small ( $23.40 \pm 2.77$  mm) sizes. These scallops were cultured in trays at a stocking density of 80 large scallops/tray and in bags containing 275 large specimens and 300 small scallops per bag. Large queen scallops were grown-out in trays at a stocking density of 80 queens per tray and in bags containing 100 specimens per bag. After 15 months of culture, the small-sized great scallops reached a height of  $79.64 \pm 6.18$  mm and the large specimens,  $81.63 \pm 6.30$  mm. In the trays moored in September a slower growth rate was observed from October to December. It seems that after sorting, the differences in size tend to decrease over time and level off at the end of the culture period. There were no mortalities for the first 12 months, whereas the last 3 months exhibited a very low mortality rate (2%). The small specimens of the great scallop cultured in bags attained a height of  $57.91 \pm 4.12$  mm and the large specimens measured  $62.50 \pm 3.22$  mm, after 15 months of culture. Mortality was very high at the end of the culture, 68% and 66% for large and small specimens respectively. The large *Mimachlamys varia* scallops moored in trays had reached a height of  $49.37 \pm 2.12$  mm in April 2004, exceeding the commercial size of 40 mm. In December 2004, the date of the last sampling, they measured  $60 \pm 5.31$  mm. The mortality rate was 12%. Growth in bags was very good, with the large specimens having attained a size of  $43.02 \pm 3.91$  mm in April 2004, which means that they reached commercial size in 6 months. After 15 months they measured  $56.66 \pm 4.27$  mm and total mortality was 8.72% at the end of the culture. The small specimens of this species took slightly longer to reach commercial size (9 months) and their mortality was 10%. The scallops were hung on ropes and placed in bags to be able to observe the number of specimens that settled. Settlement was only average—just half of the scallops settled on the ropes. In contrast, the growth of the animals that did attach to the ropes was good, and the mean height of the scallops hung on ropes increased from  $12.83 \pm 2.27$  mm to  $48.25 \pm 3.12$  mm. The scallops grew 35.42 mm in 11 months. In

the floating trays there was no mortality except in the spat removed in August, during which time it reached practically 100%, probably owing to the great fluctuations in temperature that were recorded. The spat grew quickly in the trays during the first winter, attaining a height of  $40 \pm 2.51$  mm in March. In summer the growth rate started to diminish, with the average height in November being  $52 \pm 4.82$  mm. Starting in October, mortality decreased and the spat grew rapidly in the bags. Average spat height went from  $16.6 \pm 3.47$  mm when they were detached in September to  $28.8 \text{ mm} \pm 3.76$  in January, with a mortality of 37.5%. The three species grew more rapidly in the trays than in the bags, although it is still too early to draw conclusions. In any case, the bags are easier to handle and less expensive than the trays and spat can probably be held there for a period of time before being transferred to tray culture. When the trays and bags containing *Mimachlamys varia* and *Aequipecten opercularis* were removed in November, 2003, a high mortality was detected, coinciding with a high mortality of adult *Mimachlamys varia* being cultured in other trays. This mortality might be attributable to some type of viral or bacterial infection. There also could have been a problem with handling as the spat might have been out of the water for too long, in addition to the high temperatures during the month of September when the scallop spat was being removed. The scallops of *Mimachlamys varia* that did survive underwent excellent growth and presented a very low mortality rate. The prospects for the use of collector bags and rope hanging techniques in scallop culture look very promising. The hanging technique needs to be improved, as it is a process that requires skill. Also a longer-lasting mesh should probably be considered for use.

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**SCALLOP SPAT PRODUCTION IN NORWAY—IS LAND-BASED NURSERY NEEDED AS AN INTERMEDIATE STEP BETWEEN HATCHERY AND SEA?** Gyda Christophersen and Thorolf Magnesen. Centre for Studies of Environment and Resources, University of Bergen, PO Box 7800, 5020 Bergen, Norway

Traditionally small *Pecten maximus* spat (post-larvae) of approximately 2-mm shell height have been directly transferred from the hatchery to sea-based nursery. Post larvae are obtainable on a year round basis, whereas spat production depends on environmental conditions in the sea. Spat size and timing of deployment are found to be determining factors to ensure high spat yield (Grecian et al. 2000, 2003, Christophersen & Magnesen 2001, Rupp et al. 2005), and the growth season in Norwegian waters is limited by

low sea temperature in the spring (5 °C to 10 °C). Strategies to extend the production period have been pursued by acclimating small spat to colder water (Christophersen & Magnesen 2001) and by using solar heated "poll" as water source to land-based indoor nursery (Christophersen & Lie 2003). The occurrence of potential predators, which can settle inside the equipment and fouling are other factors that may impair the yield (Louro et al. in press). During the warm water season deployment of spat may coincide with the settlement of starfish larvae, and presence of *Asterias rubens* have caused heavy losses. With the aim to improve survival of spat deployed to the sea, the scallop hatchery in Norway (Scalpro AS) built an outdoor land-based raceway nursery, as an intermediate step between the growth to 2 mm in the hatchery and final nursery growth in the sea from 6–10 mm to 15–20 mm. Different trials have been undertaken to investigate the performance of spat in order to evaluate the usefulness of the new land-based nursery. The spat were obtained from *P. maximus* broodstock collected from natural scallop beds in Hordaland County, western Norway, which was conditioned and induced to spawn according to standard methods at Scalpro AS. Small spat (1–7 mm) were transferred from indoor conditions in the hatchery to an outdoor land-based nursery after settlement either on downwelling screens or on collector bags. Spat that had settled on screens were relayed onto trays, whereas the collector bags with attached spat were transferred directly to the nursery. Growth of spat of different size at transfer was compared, as were spat grown in the land-based and the sea-based nursery. The spat allowed to settle on collector bags had been exposed to different tank conditions (light, water-circulation, bag color) and the subsequent yields were examined. The land-based nursery was of a flow-through raceway type with 10 parallel raceways of length 10 m, width 0.6 m and depth 0.8 m, which could potentially hold 7 million small spat. Seawater was filtered through a drum filter of 100-µm mesh and supplied the nursery at a pumping rate of 6 m<sup>3</sup> per minute, equivalent to a velocity of 2 cm per second. The experimental nursery period was from May to September when the sea temperature was 7°C to 19°C. Preliminary results showed that growth in the land-based nursery was equal to growth in the sea considering spat that retained on 6 mm mesh after 6 and 9 weeks growth. The relative shell-height growth was size dependent and varied from 1.5% to 4.2 % per day. On land, 85 % of spat of initial size 1.4 and 1.8 mm survived, whereas survival of spat of initial size 3, 5 and 7 mm was 78%, 88% and 91% respectively. Survival of the slowest growing fractions was superior in the land-based nursery. The yield of spat >5 mm from collector bags varied from 46 to 2,780 per bag. In addition a large portion of spat were recovered from the bottom of the raceway. No significant differences in spat yield were found between the different pre-transfer treatments. A higher yield related to competent larvae was obtained from screens than from collector bags, and the settlement on each collector bag was too low to utilize the maximum capacity of the raceway. Without

improving the settlement per bag, the production in trays seemed to be the most viable method. Only light fouling (clogging) was observed on the trays in the raceway and starfish larvae rarely occurred. Thus it was shown that fouling organisms and predators were efficiently removed by the filter at the water intake to the raceway and that survival was improved in the system. By securing favorable growth conditions for the slow growing individuals the final spat yield may be enhanced. Maintenance of trays from the land-based nursery was less labor intensive than of trays from the sea-based nursery, but the capital cost of the land-based nursery was higher. Nevertheless, the cost difference could be compensated by the higher survival in the land-based nursery, and taken into consideration, the increased survival by deploying larger spat to sea; the use of an intermediate nursery seems advantageous.

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**IN ÎLES-DE-LA-MADELEINE (QUÉBEC, CANADA), CAN SEA SCALLOP (*PLACOPECTEN MAGELLANICUS*) JUVENILE LOSSES BE REDUCED DURING THE YEAR THEY STAY ON COLLECTORS TO REACH AN APPROPRIATE SIZE FOR HANDLING?** Georges Cliche,<sup>1</sup> Carole Cyr<sup>1</sup> and Denyse Hébert.<sup>2</sup> <sup>1</sup>Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec, Direction de la recherche scientifique et technique, 184 Chemin Principal, Cap-aux-Meules, P.Q., Canada, G4T 1C6; <sup>2</sup>Pétoncles 2000, 55 route 199, Fatima, Canada G4T 2H6.

In Îles-de-la-Madeleine (Québec, Canada), scallop producers have to deal with very high spat mortalities (>70%) during the year spat has to stay on collectors to reach an appropriate size to be

handled and put into pearl nets for intermediate culture. Usually collectors are immersed at the end of September. Recuperation and cleaning operations start in the middle of September of the following year and last until the end of November. Mortality occurs mainly between the middle of August and the end of November. Main factors pointed out to explain this phenomenon are related to the overabundance of organisms (scallop and undesirable species) inside the collectors. As time goes on, packing of organisms on the bottom of the collector bag increases inducing very high densities of animals and their mortality. On the other hand, as organisms grow, weight of collectors increases. Longlines crash on the sea bottom and collectors drag the bottom contributing to additional mortality. Recuperation and cleaning of collectors before the middle of August, present 3 major problems: (1) scallops are still very small at that time (around 5 mm); (2) air temperatures are relatively high to handle sea scallops and (3) intermediate culture is realized in lagoons where heavy mussel fouling on the pearl nets may take place until the beginning of September. Discussions with Japanese scallop producers in 2002 brought us to test their strategy and to put very small scallops in intermediate culture at the beginning of August before high mortality affect scallop spat on commercial collectors. Because it was very important to develop a production strategy usable by industry, commercial scallop producers were associated with all steps of the experimental project. Undesirable species, mainly blue mussel (*Mytilus edulis*) and hiatellas (*Hiatella arctica*), are very abundant on collectors and sorting is required when collectors are cleaned. It is essential for producers to use a mechanical sorting machine able to handle and to sort large numbers of animals. The first challenge with the new strategy was to know if mechanical sorting was possible and effective with very small animals. A preliminary essay permitted to conclude that it was possible. For experimental trials, manual sieves were used but these sieves were adaptable to the mechanical sorting machine. In the preliminary essay of 2003, scallops were lost because adjustments of sieves were not optimum. Improvements were made in 2004 and the recuperation rate improved. Japanese producers, after having sorted manually, put small scallops in large pearl nets (50 cm × 50 cm) with a mesh of 1 mm at very high densities (up to 10,000/pearl net) and let animals grow for 1 to 2 weeks before reducing densities. They usually manipulate rearing structures many times to finally bring scallops to optimal densities for suspension culture. To reduce the number of rearing structures, we also decided to use initially high densities in a standard pearl net doubled with 1-mm mesh size net. However, to lower the manpower costs associated with reduction of densities, we decided to test 2 strategies:

- to keep the animal at high density (around 1,500/pearl net) for 2 to 3 weeks, then
- to reduce densities and to test 4 densities (200, 300, 400 and 500 scallops/pearl net 2 mm mesh size) and finally 1 month later to reduce the density to 115 scallops/pearl net for the rest of the

intermediate culture period to leave animals at high densities (around 1,500/pearl net) for 1.5 months and then to reduce densities to 115 scallops/pearl net.

Keeping in mind possible fouling problems with mussels on rearing structures in the lagoon, strategy 1 was also tested in the open sea on a site where mussel fouling could be low. On the 20 collectors used for experimental essay, the number of living scallops was estimated to be 28 180 scallops and their mean size was  $4.5 \pm 0.9$  mm. From this number, sorting permitted to recuperate and to put in pearl nets at high density, 15,051 scallops (53.4%). When pearl nets were recovered for strategy 1, 2 weeks later, 93, 3% of scallops were alive, or 49.9% of number originally present on collectors. Their mean size was  $6.8 \pm 1.3$  mm. Survival rates obtained after 1 month in pearl nets at 4 densities were very high varying between 91% and 96.5% for the pearl nets immersed in the lagoon and 91% and 93% for the ones immersed in the open sea. There was no difference between survivals obtained with different densities. Growth rates obtained with the strategy 1 were particularly interesting. In the lagoon, daily growth was 0.29 mm/day and in the open sea, 0.27 mm/day. No difference was observed between growth rates with different densities. During the same period, the growth rate of scallops left on collectors in the open sea was 0.08 mm/day. So at the middle of September, the size of scallops on commercial collectors in the open sea was 8.5 mm. The size obtained at the same time with strategy 1 was 17 mm and with strategy 2, 13 mm. With strategy 2, the recuperation rate obtained 48 days after immersion in the lagoon at the density of 1,500 scallops/pearl net was of 89% (48% of initial number found on collectors) and daily growth was of 0.18 mm/day. Mussel fouling was almost inexistent on the pearl nets and never represented a problem. The main problem associated with the mussels was that some mussels were put in pearl nets with scallops because sorting could not be totally effective. Even if the number of mussels was very low, many scallops were found trapped in byssus only 2 weeks after being put in the pearl nets and higher mortality and lower growth were associated with agglomerations of scallops and mussels. In 2004 new trials were realized. Recuperation rates were improved by 20% and the results on growth and survival were comparable with the ones of 2003. Mussel fouling was still negligible on rearing structures. This new strategy has permitted to increase recuperation rate of scallops present on commercial collectors. Treating collectors before high mortality that starts at mid August has at least doubled the recuperation rate. Sorting of small scallops at the beginning of August is possible and effective. Even if operations took place in a period of the year where air temperature may be over 20 °C, survival rates were very high. Increase in size is also very interesting because young scallops put in pearl nets earlier in the season are approximately 10-mm larger than the ones left on collectors at the middle of September. Usually scallops are seeded in May and June of the following year and this increase in length represents an advantage to improve the resistance of

scallops to predators. Scallop producers plan to realize in 2005 a pilot project to confirm the interest of this new strategy on a commercial scale.

**MANAGEMENT OF COMMERCIAL SCALLOPS IN THE BASS STRAIT CENTRAL ZONE SCALLOP FISHERY, AUSTRALIA.** Elizabeth Cotterell and David Johnson. Australian Fisheries Management Authority, Box 7051, Canberra BC ACT 2610.

Commercial scallops (*Pecten fumatus*) have been fished in the waters of Bass Strait since the 1970s, as boats explored new grounds. The major fishing grounds off Lakes Entrance in eastern Victoria, the northern coast of Tasmania, King Island in western Bass Strait and the Furneaux Island group in eastern Bass Strait have subsequently been fished heavily, with fishing activity peaking in the early 1980s at a total catch of nearly 12,000 tons shell weight (Young & Martin 1989, Zacharin 1990). At this time fishing was virtually unregulated and there was a huge increase in fishing effort and expansion of the fleet, with a trebling of effort in the fishery in the 2 years from 1981 to 1983 (Zacharin 1990). Three fisheries for commercial scallops, managed by 3 management agencies (the Victorian Government, Tasmanian Government and Commonwealth Government), evolved though all indications are that it is a single stock of scallops. Each management agency has different governing legislation and has a different management regime (later). The overall fishery in Bass Strait has been characterized by a series of booms and busts. There was a major decline in scallop catches in 1985 to 1987, which led to the closure of the Tasmanian fishing grounds from 1988. The Commonwealth Central Zone was closed in 1990 for 1 year because of low stock levels. The Tasmanian grounds reopened to fishing in 1995 but were closed again in 1997 and 2000 to 2002. The Central Zone was closed again in 1999 following dramatic declines in catch from 1997 to 1998 and did not reopen to commercial fishing until 2002. Virtually no fishing took place in that year. In 2002 the Commonwealth legislated that at least two areas of the Central Zone fishery (one in the west and one in the east) be closed to fishing each season to minimize the probability of recruitment overfishing. Since that time an area off Flinders Island in the east of the fishery has been closed to fishing. This closure is reviewed annually in the light of available information on stock status. Additional measures to protect spat and juveniles from the impacts of fishing were also implemented; a bed is not opened to fishing unless less than 20% of commercial scallops in the bed are above the minimum size limit.

The most important aspects of the various spatial management regimes used by each management agency were summarized by Haddon et al. (2004) as follows:

Tasmania: Most scallop beds closed, with small areas open, with a flexible maximum catch allocation per quota unit within the fishery and size and trashing rate limits;

Commonwealth: Most scallop beds open, with small areas closed, with an effective total allowable catch (TAC) and size and trashing rate limits;

Victoria: All beds open every year with an implied TAC. There is scope for temporal closures depending on condition and size of scallops.

One of the major challenges for fisheries management in regards to Bass Strait scallops is management of a single stock by multiple jurisdictions. The Australian Fisheries Management Authority (AFMA) was established under the Fisheries Administration Act 1991 and manages fisheries under the Fisheries Management Act 1991 (the FMA). AFMA is the Commonwealth statutory authority responsible for ensuring the sustainable use and efficient management of Commonwealth fishery resources on behalf of the Australian community and key stakeholders.

The FMA requires AFMA to pursue 5 objectives. In brief these are:

- efficient and cost-effective fisheries management
- ecologically sustainable development
- economic efficiency
- accountability
- cost recovery

AFMA must also seek to ensure that Commonwealth fish stocks are not over-exploited. AFMA's management philosophy involves a partnership approach to the management of marine resources under its jurisdiction. This approach provides opportunities for stakeholders to have input into the management process through various management advisory committees or MACs. AFMA's board of directors makes decisions on fisheries management issues based on advice from MACs. AFMA staff and other special committees. Scientific surveys have been conducted in the Central Zone since 2000. These surveys have provided information on scallop size and abundance, and have been considered by the MAC and the AFMA board when recommending fishery closures and TACs. The fishery is currently considered to be overfished (Bureau of Rural Sciences, 2003). At present there is an area closed off Flinders Island. This contains the only known bed of commercial-sized spawning scallops in the Central Zone. The most recent scientific information (M. Haddon, pers. comm.) indicates that there may be fewer than 1,000 tons of scallops present in the survey area. Scallop operators are facing some marketing challenges. These relate mainly to continuity of supply of fresh product to domestic and export markets and have been exacerbated by competition with cheaper imported product. There are also issues of overcapitalization in the fishery. Whereas the mechanism for structural adjustment exists under the management plan for the fishery, the depressed economic situation in the fishery means that there is no money available for this to occur.

The major challenge for AFMA is how to ensure an ongoing, sustainable, resource while providing an economic return to operators. Other challenges are as follows:

- Integrating spatial management and conservation objectives across jurisdictional boundaries
- Developing decision rules, e.g. for alternative season dates
- Cost-effective gathering of data on the fishery and stocks over large areas

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**IMPROVING QUALITY OF SEA SCALLOP SPAT COLLECTION BY A MORE ACCURATE DETERMINATION OF THE IMMERSION PERIOD OF COLLECTORS IN ÎLES-DE-LA-MADELEINE, QUÉBEC, CANADA.** Carole Cyr,<sup>1</sup> Georges Cliche,<sup>1</sup> Gaston Desrosiers<sup>2</sup> and Bruno Myrand.<sup>1</sup> <sup>1</sup>Station maricole des Îles-de-la-Madeleine, Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec, 184 Chemin Principal, Cap-aux-Meules, G4T 1C6; <sup>2</sup>Institut des sciences de la mer de Rimouski (Ismer), Rimouski, Québec, G5L 3A1.

Overabundant settlement of undesirable organisms on scallop collectors can negatively affect survival rate and growth of young scallops, increase time to handle and clean collectors, slow sorting operations in the plant and increase production costs. In 1998, predation by starfish (*Asterias vulgaris*) inside collectors resulted in high spat mortality. In 2001, overabundance of bivalves *Mytilus edulis* and *Hiatella arctica* in collectors has caused high mortalities. Many collectors dragged the sea bottom because the weight of long lines was too high. A study done in 2003 to 2004 near a commercial site of spat collection, located east of Îles-de-la-Madeleine (Québec, Canada), aimed to evaluate if it were possible to target more accurately the immersion period of collectors to reduce abundance of undesirable species and maintain high collection of scallop spat. Specific objectives were (1) to determine the spawning period of sea scallops and (2) to evaluate if larval monitoring and/or postlarval monitoring for peak settlement could be used as tools to determine an optimal immersion period of spat. The sea scallop spawning (length >90 mm) was investigated by monitoring the gonadosomatic index (GSI). This index was calculated as the percent ratio between the wet weight of gonad and the one of remaining soft parts. Weekly samples of 20 adult scallops (for a ratio of 50% female and 50% male) were collected by

dredging at the depth of 28–30 m, between the end of July and the middle of September on the Chaîne-de-la-Passe (fishing area) and on the Fond du Sud-Ouest (natural bed closed to fishing since 1991). To determine larval densities, a sample of known volume of seawater was pumped between 2 and 8 m off the bottom at three sites. The larval monitoring was realized between July 4 and October 29. Larvae of sea scallops (*Placopecten magellanicus*) and 4 undesirable species (molluscs: *Mytilus edulis*, *Hiatella arctica*, *Anomia* spp. and echinoderm: *Asterias vulgaris*) was identified and counted. A random sub-sample of 30 larvae of each species was also measured under a stereomicroscope analyzer Bioquant IV Leica (×100). Spat collection monitoring (2nd tool): Recruitment of newly settled sea scallop and undesirable spat species was estimated using artificial collectors immersed for 7-day intervals during the scallop postlarval settlement period. Collectors were made of four Netron sections inside a 2-mm mesh spat-collecting bag. Collectors were suspended at 2 m off the bottom. Collectors were immersed weekly at three sites (water depth from 30–32 m), for 11 consecutive weeks, starting on August 19. A week after deployment, collectors were retrieved and cleaned over a 250-µm mesh sieve. The content of collectors was preserved in 95% ethanol. Sea scallops and four undesirable spat species found on collectors were counted. A random sub-sample of 30 specimens of each species was also measured. In 2004, to verify loss of spat during collection, sleeves of 250-µm mesh were gently placed over the collectors underwater before their retrieval. Other spat collectors made as described earlier, were placed in the commercial spat collection site at 2 m off the bottom. Five collectors were deployed weekly (water depth from 30–32 m), for 6 consecutive weeks, starting September 15. In December 6, collectors were retrieved and cleaned. The content of each collector was preserved in 95% ethanol and analyzed as described earlier. The GSI monitoring showed that adult scallops initiated spawning by August 26 on two sites sampled. Spawning was completed on September 15. Larvae of mussels were most abundant. Highest larval density of 15.96 larvae/L (>200-µm shell height) was recorded on September 2. Other species had densities lower than mussels. We observed a peak of larvae of *Anomias* on October 20 (2.71/L), of *Hiatellas* on October 1 (0.45/L) and of sea scallop on October 29 (0.10/L). Of the undesirable organisms, mussel was the most abundant. Highest density (1148 spats/collector) was observed on August 26. Other molluscs in order of decreasing abundance included: sea scallop (465/collector on October 20), hiatellas (354/collector on October 26) and anomias (47/collector on September 8). Starfish, a major predator of scallop spat, was only present in low numbers (between 0.33 and 6/collector) except on September 22 with 26/collector (diameter between 0.8 and 1 mm). In 2004, use of divers to recuperate a sub sample of collectors inside bags with mesh of 250 µm showed that there was no loss of organisms when collectors were recovered directly from the boat. We can observe a peak of scallops in collectors immersed on October 7 (6147/collector). Highest densities of mussels, *Hiatellas* and *Anomias* were found on col-

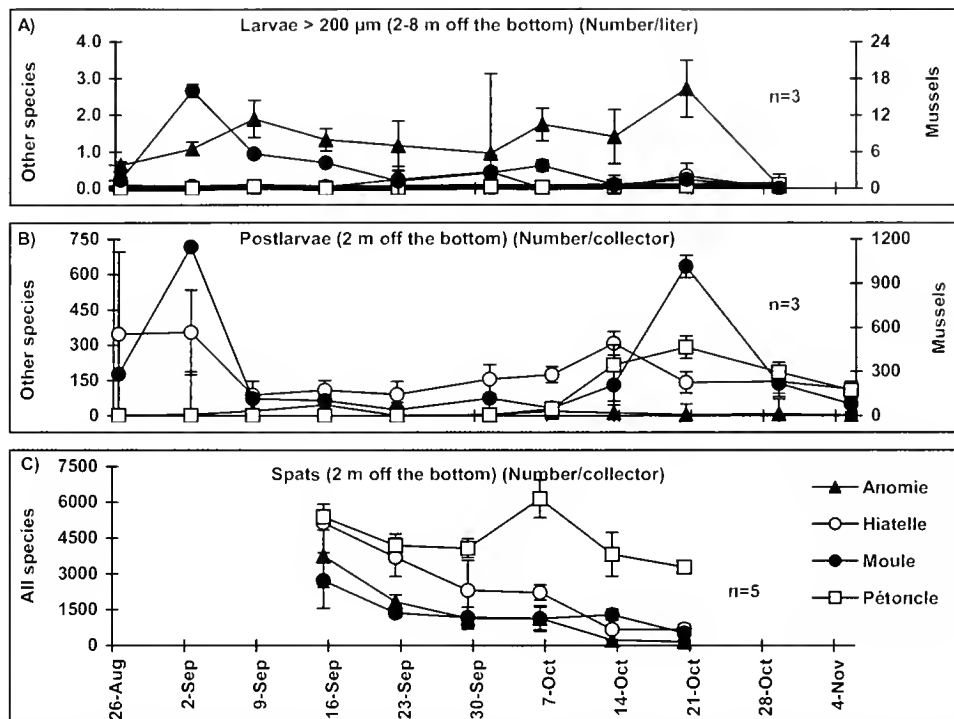


Figure 1. A) (First tool) Number of larvae per liter pumped between 2 and 8 meters off the bottom  $\pm$  ecartype (scale for mussels at right), B) (Second tool) Number of postlarvae per collector immersed one week  $\pm$  ecartype (scale for mussels at right). Collectors were immersed and retrieved a week later. C) Number of spats per collector immersed to different periods  $\pm$  ecartype. Collectors were retrieved on December 6, 2003.

lectors immersed on September 15 with respectively 2727, 5154 and 3760/collector. Collectors immersed 8 weeks after the beginning of spawning of sea scallop (October 21) contained 3281 scallops, 532 mussels, 683 *Hiatellas*, 165 *Anomias*. Starfishes were found in low numbers on collectors in 2003 (only 1 on September 15 and October 13). Monitoring of larval abundances permit to identify peak of larvae >200- $\mu$ m but aside from mussel, it does not seem to have a relation between larval abundance and spat settlement in collectors. For mussels, the curves present the same tendency to decrease with time (Fig. 1A and 1C). Scallop larvae were always the less abundant, whereas this species was always the one most abundant on collectors (Fig. 1A and 1C). With results of mussel larval monitoring, it would have been possible to recommend to scallop growers to start immersion of their collectors on September 23. The number of mussels would have been reduced by half still with a good scallop collection rate. The weekly monitoring of spat collection is a good tool to identify peak of settlement. The high collecting rates of mussels and hiatellas were observed at the end of August and at the beginning of September (Fig. 1B and 1C). Results of weekly monitoring of spat collection would not have been useful to reduce the number of mussels and hiatellas on commercial collectors deployed 3 weeks after the scallop spawning. Some years, the first peak of the collection of mussels and hiatellas may be later in September. When it happens, weekly spat monitoring could be a useful tool to identify the prob-

lem and to recommend to producers to slightly delay the immersion of scallop spat collectors. Another useful utilization of these results is the possibility of knowing if there is still interest in immersing commercial collectors late in the season. Meteorological conditions are particularly rough in fall and it is not always possible for producers to immerse their collectors in a short period of time. Aside from mussel, weekly monitoring seems to underestimate the collecting success of scallop, *Hiatella* and *Anomia*. A possible explanation of low collecting success of certain species is that collectors immersed for 1 week are not fully effective for certain species possibly because conditioning of collecting substrate (bacterial biofilm) is not optimal. Other experimental works will be necessary to validate this hypothesis.

**VIDEO CAMERA SURVEY OF WILD SEA SCALLOP BEDS VERSUS ENHANCED BEDS IN THE SOUTHERN GULF OF ST. LAWRENCE.** Leslie-Anne Davidson and Monique Niles, Dept of Fisheries and Oceans, Leslie-Anne Davidson, Dept of Fish. and Oceans, 343 Université Ave, Moncton, N.B. E1C 9B6 Canada.

In the southern Gulf of St. Lawrence, sea scallops (*Placopecten magellanicus*) can be found on various beds. This resource has been commercially fished since the turn of the century, and in the

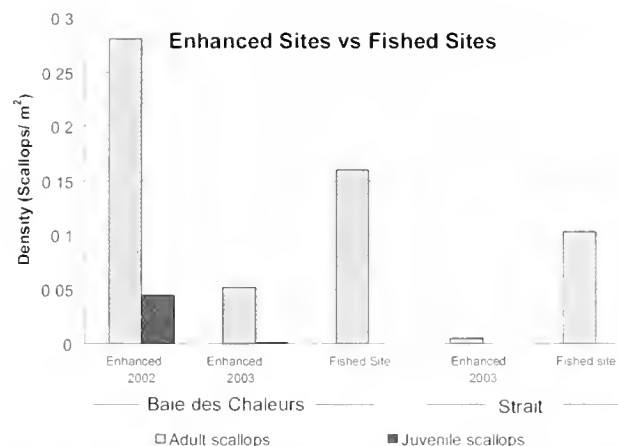


Figure 1. The density of adult and juvenile scallops on the enhanced and fished sites.

last few years the scallop landings have decreased. The Maritime Fishermen's Union (MFU) in New Brunswick Canada has been involved in a scallop enhancement project since 1996. However an effective enhancement program has only been in place since 2000. This study attempts to evaluate the success of the enhancement activities by comparing enhanced sites to fished sites. In October 2003, a study was launched to compare the scallop density of enhanced beds to commercially fished beds. An underwater video camera mounted on a sled was towed from a research vessel over the bottom at a speed of 1 knot. Other equipment required for the study included a television, digital recorder, cassettes, global positioning system (GPS) and a computer. The camera was oriented to video tape the marine bottom, and the geographical position was recorded using a GPS overlay. Each site was video taped for 3 to 4 hours. Video recordings were analyzed to determine the density of the adult scallops, the swimming juvenile scallops, sea stars, crabs, lobsters, other species and bottom type. The survey was conducted over three enhanced beds and two commercial fished beds (Table 1).

TABLE 1.  
Scallop beds evaluated using video.

Site	Date Seeded	Area Evaluated (m <sup>2</sup> )	Date Evaluated
Fishing sites: Northumberland Strait	N/A	7752	October 1, 2003
Baie des Chaleurs	N/A	8640	October 8, 2003
Enhancement sites: Northumberland Strait	May 2003	10932	October 2, 2003
Baie des Chaleurs	May 2002	3372	October 7, 2003
Baie des Chaleurs	May 2003	7992	October 7, 2003

The adult scallop density on the enhanced site that was seeded 18 months before the video survey was 1.7 to 2.7 times higher than those on the fished sites. Conversely, less adult scallops were recorded on the enhanced sites that were seeded only 6 months prior to the survey (Fig. 1). The young swimming juvenile scallops were observed on all sites except on the fished site in Baie des Chaleurs (some juvenile scallop density values are too low to be seen on the graph). The highest density of juvenile scallops was observed on the site seeded 18 months before the video survey. The scallops seeded 6 months before the survey are possibly too small to be observed by the video camera. Nadeau and Giguère (2000) reported that density assessments using the video camera evaluation are only 75% accurate. However, positive results of the enhancement project seem to be measurable 18 months after the seeding. Nonetheless, the scallop density on the enhanced and fished sites in the southern Gulf of St. Lawrence is low relative to enhanced beds in other parts the world. Hébert and Nadeau (2002) reported that in New Zealand the aim is to harvest an enhancement site when the density has reached to 1 scallops/m<sup>2</sup>.

Data regarding the density of all species at the study sites indicated that seastars are found in higher numbers on the enhanced sites than the fished sites (Fig. 2). Because the seastars prey

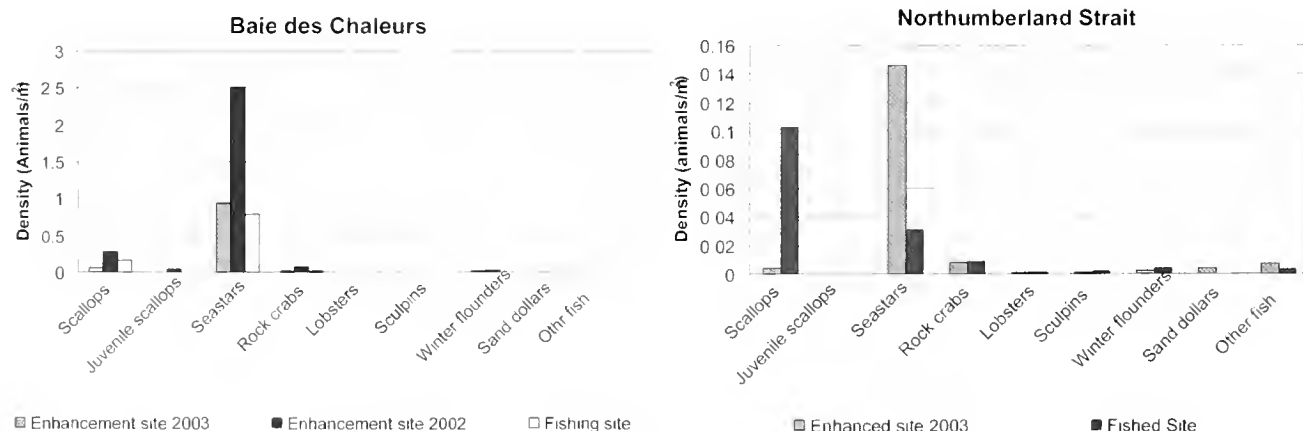


Figure 2. The density of all species on the enhanced and fished sites.



on scallops their presence may have a negative impact on the success of the project.

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## BREEDING SEASON OF *CHLAMYS VARIA* (L.) IN SPAIN.

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The black scallop *Chlamys varia* is being considered as a candidate for aquaculture in Spain. There are no dense local populations of this species in Spain, and no commercial fishery has been developed. Two areas of Spain show promising conditions for culture of this species; in Galicia (NW Spain, Atlantic Ocean) the black scallop was formerly fished, but nowadays landings are no longer recorded and supply of spat depends on hatchery production. In Málaga (South Mediterranean), black scallops are not commercialized, in spite of the abundant settlement found to occur in collectors. Settlement in collectors and spawning in hatcheries are directly linked to the breeding cycles, therefore a detailed knowledge of the cycle in both areas is important for aquaculture management. The breeding cycle of *C. varia* has been previously reported for France, Isle of Man, Ireland and Galicia. As a rule, the breeding cycle shows a bimodal pattern. The timing of the cycle varies with latitude, with the breeding season taking place later in northern populations. Reddiah (1962) recorded spawning in Isle of Man in June and November, whereas Burnell (1983) reported spawning in June and August on the west coast of Ireland. In French waters spawning takes place in May and September (Lubet 1959, Lucas 1965, Shafee & Lucas 1982). According to Parada et al. (1993) in Galicia *C. varia* spawns in March, May and August. There are no reports available about the breeding season of *C. varia* in Málaga. According to previous reports, *C. varia* is a protandric consecutive hermaphrodite in which the gender ratio is related to age/size, with a higher percentage of females being recorded in older/larger scallops. Gonad color is variable but is often a whitish-cream color, regardless of gender, although sometimes a yellow gonad has been recorded and identified as female (Lubet 1959, Lucas, 1965). The objectives of this study are to compare the breeding cycle and the sexual behavior of populations of *C. varia* from Galicia and Málaga. Hatchery produced spat of *C. varia* (Galicia) and spat settled on collectors in Málaga were main-

tained in cages suspended from a raft (Ria de Arousa, Galicia) and from a long-line (Marbella, Málaga) respectively. In Galicia, the spat was produced in the hatchery in June year 0, transported to sea in autumn year 0, and maintained in intermediate culture I and II (Román et al. 2003) until February year 2 (mean size  $42.3 \pm 4.7$  mm, 21 months old), when sampling for studies on breeding cycle began. In Málaga, collectors were deployed in April year 0 and detached in November year 0. Sampling began in January year 1 (mean size  $36.1 \pm 2.8$  mm, 11 months old). In both cases, sampling was carried out fortnightly. Thirty scallops were measured, the gonads removed and the dry weight (100 °C, 24 h) obtained. Gonad color was recorded and gender determined by microscope analysis of gonad smears. The breeding season in Galicia starts in spring and goes on until the end of summer. As in northern populations, two main breeding periods were observed, with a maximum recorded between April and May. As a consequence of spawning in this period minimum values were observed at the end of May. After a rapid recovery a new maximum was reached in mid-June followed by a slow decrease in gonad weight until minimum values were reached in September, indicating the onset of the resting period. A higher proportion of males was recorded, although a slight increase in the proportion of females was recorded as the breeding season proceeded. The gender ratio (F/M) was always <0.5 between February to May (first spawning) but increased to 0.7 in the second period. Color was not useful for distinguishing the gender, because both male and female gonads varied in color between white and cream. However, some females bore yellow gonad. In Málaga the breeding season, which began when scallops were 9 months old, extended from middle winter to the end of summer. During this time continuous reproductive activity was observed with probably five spawning events. The breeding period ended by mid August. In this population, the mean value of the F/M ratio was 1.1, and the color of the gonad was useful for differentiating between males and females, because males always bore white gonad whereas the female gonad was always yellow or orange. In Galicia the breeding period was found to be similar to that recorded in other northern populations, except that gonad

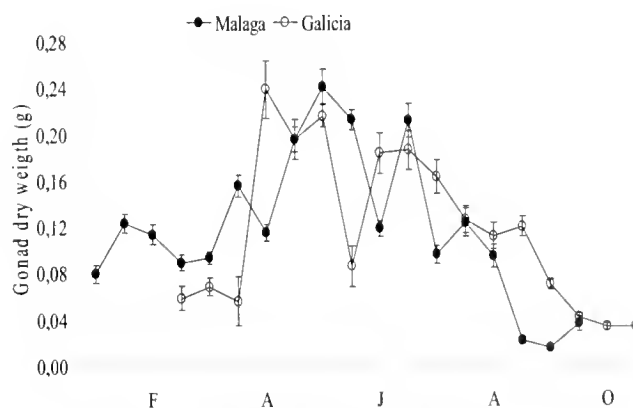


Figure 1. Breeding season in two populations of *C. varia* in Spain.

development began and ended earlier, whereas in Málaga the reproductive cycle began earlier and ended earlier and involved more spawning events than any other cycle described for this species. The two populations differed in terms of the gender ratio, gonad coloration and timing of breeding which may, to some extent, be related to environmental factors (temperature, food availability, photoperiod) associated with latitude. The Galician population behaved in the same way as the northern populations, whereas the population from Málaga showed distinctive and exclusive behavior never previously recorded in other populations.

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## SCALLOP CULTURE IN AUSTRALIA—SCOPING STUDIES, DEVELOPMENT AND REALITY. Mike Dredge, 40 Hall Road Narangba, 4504, Queensland, Australia

Scallop culture is a major form of aquaculture; one with global production now in the order of 2 million tons per year. Whereas scallop culture hasn't developed into a viable industry in Australia thus far, one Australian species, *Amusium balloti*, has growth and market characteristics that offer promise for scallop aquaculture. A detailed feasibility study on scallop aquaculture, incorporating biological, marketing and economic reviews and modeling was funded by FRDC and completed by a diverse and experienced technical working group in 2002. The economic models suggested profitability under almost all operating scenarios. Two Australian companies have attempted to develop scallop culture based upon *A. balloti* during the past 5 years. One of these companies was set up as a direct result of the feasibility study's output and the other has used development procedures remarkably similar to those described in the feasibility study. Both companies have struggled to develop their operations into economically viable businesses thus

far. Developmental delays can be attributed to both organizational and technical limitations. One company, based in Western Australia, has fully developed infrastructure and authorization, but has had problems with consistency of spat supply. The second, Queensland-based company has experienced a series of delays caused by authorization processes and staff turnover. Some of these delays can be attributed to the company's failure to fully understand government process and legislation. At the same time, government procedures for establishing marine aquaculture in a form that required exclusive access of the seabed and water above it were very poorly defined. Under capitalization and delays in establishing an appropriate organizational structure may have exacerbated the company's problems. There is a good deal to be learnt from this case study. Key points include over-optimism when dealing with technical uncertainty, assumption that private enterprise can develop effective management and new technical skills quickly, budgeting and capitalization of sunrise industries and failure of communication between government agencies responsible for licensing and private venture companies. Companies undertaking new aquaculture operations need to understand government process and need to be sufficiently capitalized to withstand process uncertainty and delay. Above all, they must have a management structure that understands the technicalities and uncertainties of the sunrise industry in question.

## POSSIBILITIES AND MYSTERIES IN SCALLOP REPRODUCTION. Arne Duinker, National Institute of Nutrition and Seafood Research (NIFES), PO Box 2029 Sentrum 5804, Bergen, Norway.

The reproduction is an interesting and puzzling part of the biology of *Pecten maximus*.

Some aspects of the main framework are relatively understandable, with clear, testable hypotheses. For the first time in bivalves, oysters have been successfully manipulated with phase-advanced regimes of temperature and photoperiod resulting in a phase advance of the winter restart of gamete development (Chavez-Villalba et al. 2002). A framework for similar manipulation of the annual reproductive cycle has been suggested for *P. maximus* (Duinker 1999; 2002), supported by evidence of a photoperiod effect on early gonad development (Duinker et al. 1999). This kind of experiment would reveal important information about the reproduction of any species of commercially exploited scallops, but still remains to be carried out. Comparative studies with different species would be particularly valuable in this context. Seasonal variation is seen in the larvae yield in hatcheries for *P. maximus*, but short-term variations lead to reduced predictability and increased efforts and number of induced spawnings (pers. comm. T. Magnesen, University of Bergen). These problems seem more complicated and more difficult to study but nonetheless interesting. The variations may be based on several aspects of the cellular physiology in scallop reproduction. Examples may include cycles of

atresia and oocyte growth (Paulet & Boucher 1991) leading to cycles in readiness for spawning and some final preparations for spawning with arrested recruitment of young oocytes and accelerated growth of the remaining cohorts (Duinker 2002). The latter has been observed in *P. maximus* as absence of young cells (Mason 1958, Duinker & Nylund 2002), and Mathieu et al. (1982) showed that oogonial mitosis in mussels was inhibited in mussels prior to spawning by a substance in the mantle tissue. This leads to the question if spawning should be induced in more than one step, with a first stimulus preparing the gonads for spawning and a final stimulus that initiates the actual spawning (Duinker 2002). A similar two-step model was described for the osphradium in *P. maximus* and *Placopecten magellanicus* (Beninger et al. 1995), where a sequence of a “ready” and a “go” cue initiates the accumulation of monoamines and subsequently their release in the gonad, initiating spawning. To increase the predictability in scallop hatcheries, a better understanding of the reproductive biology is needed. Studies of the main framework and the cellular may be necessary. Because research on scallop reproduction is scattered around the world, international cooperation with comparative studies of different species could be beneficial, in particular if involving labs with complementary expertise. One approach could be to compare a species that is relatively easy to spawn, such as the Chilean *Argopecten purpuratus*, with a species that gives more variable results as *P. maximus*.

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- INDUSTRY TRIALS OF A SEA SCALLOP DREDGE MODIFIED TO MINIMIZE THE CATCH OF SEA TURTLES.** William D. DuPaul<sup>1</sup>, David B. Rudders<sup>1</sup> and Ronald J. Smolowitz<sup>2</sup>. <sup>1</sup>Virginia Sea Grant Marine Advisory Program, Virginia Institute of Marine Science, Gloucester Point, VA 23062, USA; <sup>2</sup>Coonamessett Farm, East Falmouth, Massachusetts 02536 USA.
- In response to increasing numbers of sea turtle interactions observed by the sea scallop industry and subsequently corroborated by NMFS observers, a series of 15 experimental cruises were carried out during the summer and early fall of 2003 and summer of 2004 on the continental shelf waters of the mid-Atlantic Bight. The objective of the cruises was to examine the efficacy of a modified commercial sea scallop dredge designed to reduce the bycatch of sea turtles in the sea scallop fishery. The modification consisted of a chain mat spanning the opening of the dredge mouth. The performance of the experimental gear was assessed by comparing a modified dredge fished simultaneously with an unmodified dredge. Results indicate that the modification was successful in eliminating the bycatch of turtles with relatively small reductions in the catch of the target species. A total of 3,078 tows in 277 days at sea was observed during the trials with eight sea turtles captured in the unmodified dredge and none captured in the modified dredge. Of the tows that were sampled by the observers, the modified dredge captured significantly ( $P < 0.001$ ) less scallops relative to the unmodified dredge. On a percentage basis, the modified dredge captured 6.8% less scallops than the unmodified dredge. It is anticipated, however, that the difference in sea scallop catches will decrease over time as industry becomes more familiar with the use of the chain configuration. These cruises demonstrated that a simple modification to the standard sea scallop dredge can be effective in eliminating the incidence of sea turtle bycatch without substantial concomitant reductions in the capture of the target species.
- DISTRIBUTION AND ABUNDANCE OF TWO LION PAW SCALLOP SPECIES (*NODIPECTEN SUBNODOSUS* AND *N. NODOSUS*) IN LATIN AMERICA.** Esteban Félix-Pico,<sup>1</sup> M. Arellano-Martínez,<sup>1</sup> G. Ponce-Díaz<sup>2</sup> and A. Massó-Rojas<sup>3</sup>. <sup>1</sup>Centro Interdisciplinario de Ciencias Marinas-Instituto Politécnico Nacional, Apdo. Postal 592, La Paz, B.C.S., C.P. 23000, México; <sup>2</sup>Centro de Investigaciones Biológicas del Noroeste, S.C., Apdo. Postal La Paz, B.C.S., C.P. 23000, México; <sup>3</sup>Centro de Regional de Investigaciones Pesqueras-Instituto Nacional de la Pesca, Apdo. Postal La Paz, B.C.S., C.P. 23000, México.
- Two species of scallops belong to the genus *Nodipecten*, the Central Atlantic Lion's Paw and East Pacific Lion's Paw scallops, *Nodipecten nodosus* (Linnaeus, 1758) and *Nodipecten subnodosus* (Sowerby I, 1835), although some authors place the East Pacific Lion's Paw scallop in a separate genus, *Lyropecten* (Rombouts, 1991). East Pacific Lion's Paw scallops are found from central Baja California peninsula (28°N) to the Peru (5°S) and into de Gulf

of California in depths of 3–50 m, generally on sand bottom. Distribution is patchy throughout the range and no extensive beds occur, only in Mexico the one largest center of population is in the lagoon Ojo de Liebre, Baja California Sur. The largest and heaviest of the West American tropical species grows up 210 mm. The other one, Central Atlantic Lion's Paw scallops occurred widely throughout the Atlantic coast from South Carolina, USA, Caribbean sea to Brazil and populations support small fisheries. They are large and can reach a shell height of 150 mm (Lodeiros et al. 1999). This study presents the knowledge regarding the fisheries of *Nodipecten* spp. mainly in the Western coast of Mexico and some references in other countries of Latin America. The analysis is based on landings statistics, biomass and population size estimated, and literature reports on the fishery and population dynamics of the scallop. This description approaches topics on the characteristics of the fishery, population changes, catch quotas, prices and local and export markets. In the capture works, approximately 152 people participated, and in the shucking process close to 135 were employed during the season 2004. Most of the catch was taken by divers using semi-autonomous diving. The capture quota per equipment was between 300–400 scallops/day, and each fishing session permits only from two to six pieces of equipment. The biomass was estimated by the swept area method, whereby samples were taken by diving at each 100–250 m and selecting sampling grid at random by CRIP researches. To increase precision in the estimate biomass we made a post-stratification, defined as a new classification of strata, using the observed catch per station. Animals of the *N. submodosus* larger than 80-mm shell height had mature gonads, indicating that sexual maturity is reached at about 1–1.5 year old. The minimum harvest size limit of most measures 140 mm. The population distribution occurs in a patchy pattern and normal densities attain one to two specimens per m<sup>2</sup> in the beds of the lagoon Ojo de Liebre. The Mexican regulation determined a closed season to protect breeding stocks between September to November, although the fishing period is only 6 months a year (May to July and November to January) (Masoó-Rojas et al. 2000). Mexico reported the greatest change from 1995 to 1999, going from 35 t of muscles to an historical maximum of 157 t valued at approximately USD\$2,072,081. The total biomass estimated per year increased during the years 1996 to 1998 from 556–1,600 t. For 1999 the total biomass fell to 1,300 t then later it increased from 770–1,140 t during 1999 to 2004. Overfishing as well as environmental changes, such as those produced by the El Niño 1997 to 1998 have caused changes in its population densities and size structure at the bahia Ojo de Liebre. This descent cannot be attributable to an illegal catch; the most probable cause would be the effects of the El Niño of 1997 to 1998. The landings increased to 100 t of muscles per year from 1996 and remained fairly constant thereafter. The home market for Lion's Paw scallop, though growing, is exported, mainly to the United States. The home markets are La Paz, Los Cabos, Ensenada, Tijuana, Cd. Mexico and the United States. Most scal-

lops are exported (more than 50%) as fresh or blast frozen. The beach price reached values of 150–160 Mexican pesos per kilogram of muscles (approx. \$13.9 USD), during the season of 2004. Minor landings of the Atlantic species *Nodipecten nodosus* occurred sporadically along the coastal points of Colombia, Venezuela and Brazil. Distribution is patchy and they are not particularly abundant in any one locality. Most of the catch is incidentally taken by shrimp trawlers. No attempts have been made to estimate population size or structure in any area. The special distribution and temporal variation of *Nodipecten* spp are in abundance in México, Venezuela, Colombia and Brazil. The lion's paw scallop is characterized by extreme dynamism. Directed fishery for this species is disproportionally dependent on a single or a few, often well spaced, year-classes. The rapid growth of L. Paw scallop, on the other hand, has been more persistent particularly in the northern aspect of the bank.

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**GROWTH IN RECOVERING BEDS OF TASMANIAN SCALLOPS (*PECTEN FUMATUS*).** Malcolm Haddon, Jayson Semmens and Julian Harrington. Marine Research Laboratory, Tasmanian Aquaculture and Fisheries Laboratory, University of Tasmania, Private Bag 49, Hobart, Tasmania 7001, Australia.

Stratified random dredge surveys demonstrated that Tasmanian scallop beds and the fishery reliant on them collapsed over the 1999/2000 period. The series of annual surveys (at least) that followed this event provided the opportunity to detect and follow the growth of particular cohorts in scallop beds as they re-established themselves. Beds of different densities established themselves in different regions and their growth characteristics were different. The only scallop beds surveyed in the State of Tasmania's waters were all relatively dense. Conversely, the only scallop beds found in Australia's Commonwealth waters near Tasmania were of relatively low density, at least up until 2004. It was only the discovery of a very dense scallop bed in Commonwealth waters in 2004 that permitted a more thorough comparison of growth rates across geographical areas and density. The stock collapse meant that new cohorts could be identified and their growth followed through time. Modal analysis was used to characterize the size distributions of scallops sampled off the beds. Comparison of the relative growth rates of scallops from different areas and densities indicated that density of scallops influenced growth rates, especially after reaching between 70 and 80 mm shell

length. Such variations in growth rate have significant implications for stock recovery times and thereby any fishery, based on the stocks. The minimum time to reach the legal minimum length provides an indication of the minimum number of alternative scallop beds that need to be available to any rotational or paddock harvesting regime.

**COMPARISON OF DREDGED BENTHIC FAUNA FROM AREAS OF DIFFERENT FISHING INTENSITY—EXAMINING THE EFFECTS OF FISHING DISTURBANCE.** Julian Harrington, Jayson Semmens and Malcolm Haddon. Marine Research Laboratories, Tasmanian Aquaculture and Fisheries Institute, University of Tasmania, Private Bag 49, Hobart, Tasmania 7001, Australia.

Vessel monitoring system (VMS) data from the 2003 Tasmanian and Commonwealth Central Bass Strait commercial scallop (*Pecten fumatus*) fisheries, Australia, was used to define 500 m × 500 m areas that were impacted by different levels of dredge fishing. Dredge samples were subsequently collected from heavy, moderate and low fished areas of the Tasmanian fishery; and heavy, moderate, low, not-fished and pre-fishery (samples that were collected pre-commercial fishing but overlapped areas that would be fished during 2003) areas of the Commonwealth fishery and the data compared to test for differences in the benthic communities found in areas impacted by different levels of fishing intensity. Results from the Tasmanian fishery found significant differences in the benthic communities collected from heavy fished areas and moderate/low fished areas, while in the Commonwealth fishery, benthic communities from areas that were fished (heavy, moderate and low fished blocks) were significantly different from those from not fished and pre-fished areas. These results do not seem to be a consequence of fishing disturbance itself, but are more likely caused by pre-existing habitat and/or benthic community differences, which occur over the same spatial scale as the defined fishing intensity areas.

**EFFECTS OF SEA STAR AND CRAB PREDATORS ON SEA SCALLOP (*PLACOPECTEN MAGELLANICUS*) RECRUITMENT IN THE MID-ATLANTIC BIGHT (USA).** Deborah R. Hart. NOAA/Northeast Fisheries Science Center, 166 Water Street, Woods Hole, MA 02543, USA

This study investigated the spatial distribution and potential mortality on juvenile sea scallops (*Placopecten magellanicus*) in the Mid-Atlantic Bight imposed by three potential predator groups: the sand star *Astropecten americanus*, the common sea stars *Asterias vulgaris* and *A. forbesi*, and by the crabs *Cancer irroratus* and *C. borealis*. Both asteroid groups appear to reduce sea scallop recruitment, with significantly lower recruitment in areas where the sea stars were in high abundance. However, no significant effect of *Cancer* crabs on scallop recruitment was detected. *A.*

*americanus* was most common in deep water (>75 m), *Asterias* in shallow water (27–40 m), while *P. magellanicus* was most common at intermediate depths (50–70 m). Very high densities of *A. americanus*, no sea scallop recruitment, and low *Asterias* densities were observed in all stations with depth greater than 85 m, and in many stations between 65–85 m. *A. americanus* may be an unusual type of “keystone” predator that is capable of excluding sea scallops and perhaps other prey species by direct consumption of newly settled spat, and may be excluding *Asterias* by competition and/or predation on its juveniles.

**ROTATIONAL MANAGEMENT OF THE UNITED STATES ATLANTIC SEA SCALLOP FISHERY.** Deborah R. Hart. NOAA/Northeast Fisheries Science Center, 166 Water Street, Woods Hole, MA 02543 USA.

A general theory of rotational fishing is developed and applied to the United States Atlantic sea scallop (*Placopecten magellanicus*) fishery. Rotation generally increases biomass-per-recruit, and in the case of sea scallops, slightly increases yield-per-recruit for fishing mortalities near  $F_{MAX}$ . More substantial increases in biomass- and yield-per-recruit occur at higher fishing mortality rates. Thus, rotational management can help alleviate the effects of over-fishing, and can be considered as contributing to a precautionary approach to fishery management. Rotation increases the yield from cohorts that become of exploitable size at about the time that their area is closed, but this is in large part cancelled by the loss of yield from cohorts that reach exploitable size at about the time the area is opened. This suggests that an adaptive rotation strategy that closes an area to protect a large year-class can give greater improvements in yield than regular rotation. Simulations indicate that adaptive rotation can give further improvements in yield with less area closed at any given time. However, rotation, and especially adaptive rotation, can increase the variability of yields. Rotational management and permanent closures can cause difficulties with conventional metrics of fishing mortality that are based on the assumption that mortality is spatially uniform. Averaging over time rather than space can give a more appropriate measure of fishing mortality when this quantity varies spatio-temporally.

**STOCK EVALUATION OF KING SCALLOP (*PECTEN MAXIMUS*) FISHERY OFF THE SOUTHEAST COAST OF IRELAND USING HIGH RESOLUTION ACOUSTIC.** Antonio Hervas,<sup>1</sup> Gerry Sutton,<sup>2</sup> John Hickey<sup>3</sup> and Oliver Tully.<sup>3</sup> <sup>1</sup>Trinity College Dublin; <sup>2</sup>Coastal and Marine Resource Centre, University College Cork; <sup>3</sup>Board Iascaigh Mhara, The Sea Irish Fisheries Board, Ireland.

The king scallop fishery off the south east coast of Ireland is by far the most important scallop fishery and one of the major shell-fish fisheries in Ireland. The South East coast of Ireland has had an active scallop fishery since the 1970s, however it was not particu-



Figure 1. Image showing catch rate for four of the stations carried out during the 2002 research survey. The two main acoustically distinct sedimentary facies are shown. Dark features represent gravel lag while light represent sand cover. Catches were significantly higher over dark than over light features.

larly well developed until the 1980s when a large number of boats (approximately 10 boats of 20–30 m in length) entered the fishery. During the 1990s fishing effort increased significantly from 10 vessels and approximately 100 dredges to more than 20 vessels and over 500 dredges. As a consequence of this the fishery experienced a decline in catch rates. This decline in catches was experienced in the traditionally fished scallops beds and as a result boats now have to go to offshore waters to look for larger catches. Due to this worrying development, the South East Shellfish Association saw a need for developing stock assessment procedures for the stock in their region to provide scientific data, which heretofore the fishery was lacking. In summer 2001 a collaborative research project began between BIM (Board Iascaigh Mhara—The Irish Sea Fisheries Board) and the South East Shellfish Association for the development of stock assessment procedures of the scallop fishery off the southeast of Ireland with the aim of developing a system for man-

agement. Annual research surveys have been carried out in the area of study to map the distribution and abundance of the prerecruits and recruits scallops. Type of sediment exerts a strong influence on the distribution of scallop and the understanding of how sediment type affects the distribution of scallops has been a key issue for the annual research surveys carried out since 2002. The objective of the study is to examine the distribution of the scallop stock in comparison to sea floor sediment distribution with particular attention to the effects of small-scale sediment variability on the abundance and distribution of the stock. MBES sonar data were collected by the project team using the RV Celtic Voyager of the National Marine Institute equipped with a Simrad EM 1002. Coherent overlapping (20% to 30%) swathes of sonar coverage were generated within discrete blocks whose size and location were prioritized in order to coincide with areas traditionally fished by the scallop fleet. All MBES data were managed and post-processed using CARIS hydrographic information processing system (HIPS, CARIS, 2003). Stock assessment research surveys were conducted on board a commercial vessel. Twelve scallop dredges (six on port and six on starboard) with ring size 65 mm and tooth spacing of 44 mm were used on the surveys in order to map the distribution of commercial and undersize scallops. Dredges were towed for approximately 30 min at 2.5–3.0 knots. Research cruises have been designed based on the multibeam acoustic map. Transects were plotted in order to find variability in catch rate within small scale areas where the two dominant facies occur. The relationship between sediment type and scallops density will be investigated for different size groups and scallop beds. GIS is an essential element of study, and ArcView (V3.3) has been used to provide a common platform in which all spatial data are integrated and where analytical operations are undertaken. Tasks range from initial operational planning for survey coverage through to data integration, analysis, presentation and map production. All data are projected from WGS 84 geographic co-ordinates to a common reference frame in UTM. Tabulated point data (sediment samples, photographic locations, scallop sample tow locations) are imported via

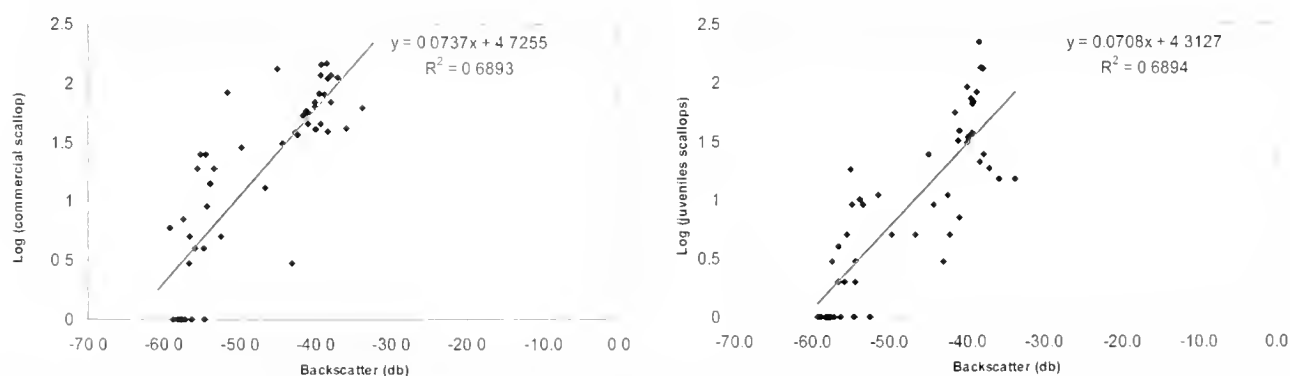


Figure 2. Log commercial scallops (left) and log juveniles scallops (right) against backscatter for 2002 research cruise. Commercial and juveniles catch rate and sediment type relationship is explained by 69%.

SQL, whereas MBES data products are imported directly. Sediment type was found to exert a strong influence on scallop catch rate (Fig. 1). Preliminary results show high relationship between the acoustic backscatter strength and density of scallops (Fig. 2).

Further analysis will investigate the relationship between sediment type and density of scallop for different size groups and different areas in order to account for those factors that can have an effect on the small-scale variability of the stock. Preliminary results show that sediments exert a strong influence on the distribution of scallops. Multibeam backscatter data can be used to produce high-resolution maps of seafloor sediment variability. The high correlation between survey catch rates and backscatter values can serve for management decisions. Information of scallop density for each of the dominant facies can be used to distribute fishing effort. The better selection of areas to fish for scallops would decrease the adverse impacts of dredging and would decrease the cost of the fishing operation. The strong links between sediment type and scallop abundance would permit to develop more precise ways to estimate scallop stock that would account for sediment-stock relationship.

**A REVIEW OF SCOTTISH SCALLOP ASSESSMENTS 1989 TO 2004.** Trevor R.W. Howell, FRS Marine Laboratory, PO Box 104, 375 Victoria Road, Aberdeen, Scotland, AB11 9DB.

The scallop (*Pecten maximus* L.) was first fished for commercially in Scotland during the 1930s and has developed into a fishery with annual landings now exceeding 10,000 tons worth £18 million. The fishery is managed by the Scottish Executive Environment and Rural Affairs Department (SEERAD). In order to manage the fishery effectively SEERAD rely on scientific advice on the state of the main scallop stocks in Scottish waters provided by Fisheries Research Services Marine Laboratory Aberdeen (FRS). Assessments of the stocks are presently carried out by FRS biennially using methods established during an internal scallop workshop held in 1990 (Cook et al. 1990) and refined during subsequent internal workshops held 1991 (Bailey et al. 1991), 1994 (Anon. 1995), 1997 (Bailey et al. 1998), 2001 (Bailey et al. 2001), 2002 (Howell et al. 2003) and most recently in 2004 (work in progress). The two main components of the assessment process are described in the presentation: numerical methods using virtual population analysis (VPA) and fishery independent surveys of the major Scottish fishing grounds. Initially an annual VPA was used to take advantage of an extending series of official landings and effort data, combined with age and length data sampled from commercial catches. In view of the pronounced seasonality of the scallop fishery the internal workshop switched to quarterly VPA in 1991. Annual fishery independent surveys to complement the VPA and to provide the most up to date information on the scallop stocks were introduced on the West coast of Scotland in 1992. By 1995 further annual surveys covering the East coast and Shetland fishing grounds were included to give comprehensive coverage of

the major Scottish scallop stocks. Biological work in support of the assessments is also discussed. The results of recent assessments are presented along with a description of current legislation in relation to scallop fishing in Scotland. In the final part of the presentation recent developments with potential for future Scottish scallop assessments are discussed. This includes a description of an effort-mapping scheme that uses scallop fishing vessel GPS data, the GSM (mobile phone) network and the Internet to provide real time location and haul data. These are used in conjunction with catch and landings data from tallybooks filled in by the vessels' skipper. Combined, these data give detailed accurate spatially ordered information on CPUE. The system, used successfully in studies on the Scottish *Nephrops* fishery is presently undergoing trials with Scottish scallop vessels.

**FOOD SOURCES OF SCALLOPS AND OTHER BENTHIC ORGANISMS—ARE ENRICHED CARBON RATIOS IN BENTHIC CONSUMERS CAUSED BY BENTHIC PRIMARY PRODUCERS?** John H. Himmelman and Marc-Olivier Nadon, Département de Biologie, Université Laval, Québec City, Québec, Canada.

We used stable isotope analysis to investigate the sources of primary productivity (phytoplankton, kelp and benthic microalgae) and the trophic structure of benthic food webs in the region of the Mingan Islands, northern Gulf of St. Lawrence. Benthic consumers were rich in  $^{13}\text{C}$ , seemingly indicating a strong dependence on  $^{13}\text{C}$ -rich benthic primary producers (kelp and benthic microalgae) as opposed to  $^{13}\text{C}$ -depleted phytoplankton as a food source. However, suspension feeders, the scallop *Chlamys islandica*, taken along a depth gradient and individuals suspended in cages away from shore were even more enriched in  $^{13}\text{C}$  than individuals on the bottom near shore, suggesting that inputs from near shore benthic primary producers are not the cause of the enriched carbon ratio in these organisms. A review of previously reported isotopic ratios from arctic and temperate regions showed that deposit and suspension feeders are almost always enriched compared to the  $^{13}\text{C}$ -depleted ratio of phytoplankton, regardless of their distance from  $^{13}\text{C}$ -rich primary producers (measured as depth). Thus, our data for the Gulf of St. Lawrence are consistent with those reported elsewhere. Factors other than the feeding on  $^{13}\text{C}$ -rich benthic primary producers must explain the carbon ratios in benthic consumers (e.g., possibly strong selective feeding on enriched particles). Finally, three distinct trophic groups characterize the benthic communities in the northern Gulf of St. Lawrence, primary producers, primary consumers (herbivores) and predators, with only a few organisms falling outside of these groups. The sea star *Crossaster papposus* and the sculpin *Myoxocephalus scorpius*, known to be top level predators, were positioned at a slightly higher level than other predators. The relatively small number of organisms located at intermediates trophic levels indicates a low level of omnivory in this system, and this contrasts with previous studies of benthic systems.



# FORCE RECORDINGS DURING ESCAPE RESPONSES OF THE GIANT SCALLOP, *PLACOPECTEN MAGELLANICUS*.

Xavier Janssoone,<sup>1</sup> Pierre Gildas Fleury,<sup>2</sup> Madeleine Nadeau,<sup>3</sup> Bruno Myrand,<sup>3</sup> Hernan Perez Cortez<sup>1</sup> and Helga Guderley.<sup>1</sup>

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Giant scallops, *Placopecten magellanicus*, respond to the presence of starfish predators with an escape response consisting of a series of rapid valve adductions that allow the scallop to jump or swim away from the predator. To evaluate the co-ordination of the activity of the tonic and phasic muscles during such escape responses, we recorded their force production by attaching a force gauge to the shell of intact scallop and then stimulating the scallop with a starfish. These recordings showed series of phasic contractions (claps) separated by prolonged tonic contractions. Numerous characteristics could be quantified from these recordings including the maximal force, mean force during the first minute, force, frequency and number of claps per series, as well as the force and duration of tonic contractions. The number of claps per series declined and the duration of the tonic contractions increased as the escape response continued. For most scallops, the rapid phasic and sustained tonic contractions reached a similar force that changed little during the escape responses. The alternation between phasic and tonic contractions suggests that periods of tonic contraction allow the phasic muscle to recuperate and facilitate subsequent phasic contractions. Principal component analysis (PCA) confirmed the co-ordination between the phasic and tonic contractions, because various characteristics of these types of contractions were closely associated.

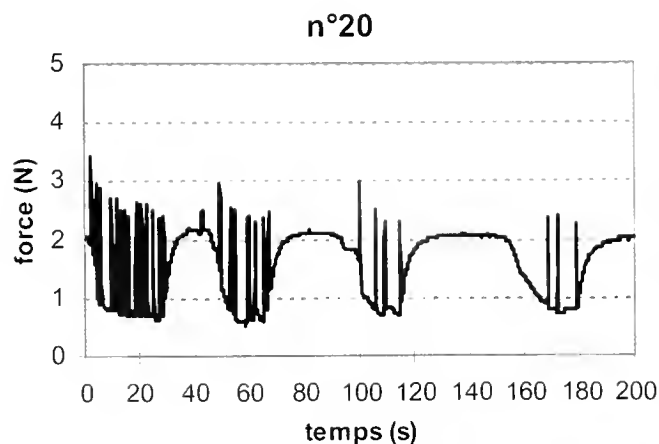


Figure 1. Force production by *Placopecten magellanicus* during stimulation with *Asterias vulgaris*. Note alternation between phasic and tonic contractions and decreases in clapping frequency as the measurement progresses.

This method combines the advantages of the simplicity of force recordings and the biological relevance of stimulation of the scallops with their predator. Patterns of force production during escape responses by individual scallops were highly reproducible, suggesting these measurements have considerable potential for tracking changes in the physiological status of giant scallops. Examination of changes in escape responses, after air exposure and after handling during mechanical sorting of scallops, is in progress.

# EVALUATION OF THE EFFECTIVENESS OF SPATIAL FISHING CLOSURES IN THE QUEENSLAND SAUCER SCALLOP (*AMUSIUM JAPONICUM BALLOTI*) FISHERY 1997 TO 2004. Eddie Jebreen, Sandra O'Sullivan, Michael O'Neill and George Leigh. Department of Primary Industries and Fisheries, PO Box 76, Deception Bay, Qld Australia 4508.

In 1996 the catch rate and total catch of scallops (*Amusium japonicum balloti*) from the Queensland scallop fishery declined markedly. In response to the decline, the Department of Primary Industries and Fisheries (DPI&F) implemented 3 permanent spatial fishing closures, referred to as scallop replenishment areas (SRA's), to reduce fishing mortality on spawning stocks and enhance recruitment to the fishery. In 2001 the area covered by the SRA's was doubled and a rotational harvest strategy developed to permit fishers access to the high densities of scallops within the SRAs. In conjunction with the creation of the closures in 1997, DPI&F established a series of annual fishery independent surveys to monitor the effectiveness of this management strategy. The performance of the SRAs and the rotational harvest strategy was assessed by investigating the relationship between scallop abundance (i.e., standardized catch rate), and closure duration (weeks closed prior to survey) using generalized linear models. The results show a significant relationship between scallop abundance and closure duration, with abundance continuing to increase for closed periods up to 4 years. This result suggests the potential of these areas to act as a source of egg production increases with duration for periods of up to 4 years.

# MANAGING SCALLOP SUSTAINABILITY AND YIELD THROUGH SPATIAL AND TEMPORAL CLOSURES IN THE SHARK BAY AND ABROLHOS ISLANDS AND MID-WEST SCALLOP FISHERIES. M. L. Joll,<sup>1</sup> M. I. Kangas<sup>2</sup> and E. C. Sporer.<sup>2</sup>

<sup>1</sup>Department of Fisheries WA, Commercial Programs, Locked Bag 39, Cloisters Square Post Office, Perth WA 6850, Australia; <sup>2</sup>Department of Fisheries WA Western Australian Marine Research Laboratories, PO Box 20, North Beach, WA 6920, Australia.

The Shark Bay scallop and Abrolhos Islands and Mid-West trawl fisheries are the major scallop fisheries in Western Australia (WA). The two areas have had a combined value of \$5–\$20 million per annum during the last 5 years. They are important fisheries



for regional WA providing employment for fishers and associated processing and support industries.

In recent years, with relatively low predicted catch and with high variability that is inherent in scallop fisheries, industry, researchers and management have looked at harvesting options and management strategies to optimize the value of scallop meat harvested in the Shark Bay and the Abrolhos Islands and Mid-West scallop fisheries. These new approaches are currently experimental. This study describes the various ways that spatial and temporal closures and fleet manipulation is used to manage the sustainability of and to optimize the yield of the saucer scallop *Amusium balloti* and how different approaches are taken in different fisheries because of the life-history traits of each area. We also discuss how spatial and temporal closures can be used to address resource-sharing issues between prawn and scallop trawl sectors, other commercial fishers and recreational fisher interests. The introduction of VMS, the relatively small number of operators and good communication links and working relationships between license holders, skippers, research and management provides for opportunities for experimental approaches in the WA scallop fisheries.

**CATCH PREDICTIONS AND ENVIRONMENTAL INFLUENCES ON RECRUITMENT OF THE SAUCER SCALLOP *AMUSIUM BALLOTI* AT THREE LOCATIONS OFF WESTERN AUSTRALIA.** M. I. Kangas, N. Caputi and E. C. Sporer. Department of Fisheries (Western Australia), Western Australian Marine Research Laboratories, PO Box 20, North Beach, WA 6920 Australia.

The saucer scallop (*Amusium balloti*) fisheries in Western Australia (WA) has shown large variations in annual catch reflecting variations in annual recruitment for a fishery and between scallop fisheries that span from Shark Bay to Esperance in southern WA. In recent years, the Shark Bay catch has varied from 1,000 to 22,000 tons, whereas Abrolhos Is. has seen a catch variation of 150 to 6,000 ton. This study describes the variation in scallop recruitment in the Shark Bay, Abrolhos Is. and Esperance scallop fisheries and explores the environmental influences on recruitment strength. The strength of recruitment is significantly negatively correlated with the major oceanographic process off the West Australian coastline, the Leeuwin Current, during the spawning season. Because the timing of the spawning season for scallop stocks in various parts of WA is different, the impacts can be variable. The possible modes of action of the current include the flushing of larvae away from suitable grounds and/or reduced spawning stimulus (also may be temperature dependent). Other possible factors affecting recruitment such as the spawning stock and other environmental variables, such as wind strength and water temperature are also examined. A good correlation has been observed from pre-season survey abundances and catches providing a forecasting capability for the main two scallop regions in WA, Shark Bay and the Abrolhos Is. This allows researchers, managers and industry to

determine the most appropriate management (e.g., starting date and duration) and marketing strategies for the season.

**RELATIONSHIP BETWEEN REPAIRED SHELL DAMAGE AND FISHING DISTURBANCE IN THE PATAGONIAN SCALLOP *ZYGOSCHLAMYA PATAGONICA*.** Betina Lomovasky,<sup>1</sup> Mario Lasta,<sup>2</sup> Silvana Campodónico<sup>2</sup> and Oscar Iribarne.<sup>1</sup> <sup>1</sup>Laboratorio de Ecología, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, Mar del Plata, Argentina; <sup>2</sup>INIDEP, Mar del Plata, Argentina.

Shell damage and abnormal calcifications are a common phenomenon in bivalve molluscs and can result from a series of interactions or disturbances that often lead to different, distinctive marks. For example, burrowing activities cause damage and chipping of the shell edge (#4, 15). Attack by crabs increases the number of scars in the shell (#16, 10), birds break the posterior margins of the valve (#9, 1, 15) or fragment the shells (#2, 3) leaving recognizable marks. Moreover, disturbances such as commercial dredging, increase the incidence of scars in the shells of *Artica islandica* (#21), *Glycymeris glycymeris* (#17), *Ensis arcuatus* (#19) and *E. siliqua* (#6,7). If properly evaluated, all these marks can be useful to understand the relative importance of different disturbance sources in different locations. *Zygoschlamys patagonica* is distributed around southern South America reaching 42°S in the Pacific to Tierra del Fuego (55°S) and 35°50'S in the Atlantic between a depth-range of 40–200 m (#5, 8, 13, 20), being an important economic resource (#12). Grounds in the Pacific occur in relatively shallow waters whereas in the Atlantic they are located along the 100 m isobath (#12). A fishery on this species started in Argentinean waters in 1996 (#12) and mainly involves four factory trawlers that process the catch on board. The fishing reached 42,969 tons scallops<sup>-1</sup> with landings of 6,018 tons meat<sup>-1</sup> during 2003 (#14). Given their importance, the identification and description of the microstructure of repaired shell damages in extant populations may be useful to understand the repairing mechanisms and infer ecological processes or possible impact of the fishery on different beds. With this purpose, we identify and describe the microstructure and frequency of repaired shell damage found in different beds and compared it with the local area swept intensity. Samplings of *Z. patagonica* ( $n = 2047$ ) were performed in 4 beds (Uruguay: 36°17'S to 53°49'W; Reclutas: 39°20'S to 56°W and 39°30'S to 55°52'W; Tango B: 42°30'S–59°05'W and Beagle: 55°10'S to 66°05'W) during 2000 and October 2004 (Beagle bed) using a total of 24 trawls. The relative intensity of commercial fishing, prior to the sampling date from 1996 to 2000, was indirectly estimated using the swept area which was calculated by considering total number of tows, speed (km h<sup>-1</sup>), towing time (minutes) and 68% of the foot rope length (#11). In TangoB bed the swept area was 448 km<sup>2</sup>, in Reclutas all sampled were taken from an exclusion fishing area (EFA) with a 0.3 km<sup>2</sup> swept area done for scientific purpose. Fishing by the commercial fleet in

Uruguay bed was related to prospecting fishing tows so a negligible disturbance of 12 km<sup>2</sup> swept area was estimated. No fishing was done in the Beagle bed. The frequency of shell damage was estimated by visual examination of the valves of all specimens. Acetate-peels technique was used to identify internal shell damage (#18). Under reflecting light, acetate peels from the polished shell cuts showed a pattern of alternating broad opaque and narrow translucent growth bands. Repaired shell damages were observed in some individuals in different parts of the valve. In general, during the process of shell repair, sediment particles were trapped in the damaged area between the old and newly accreting shell. The photomicrographs of acetate peels showed alterations in the growth band deposit, and sediment grains into the shell matrix between two growth-bands. A similar pattern of repaired shell damage is described for *Ensis siliqua* (southern Portugal) due to dredging operation (#6). The incorporation of sediment grains into the shell matrix occurs in other bivalves, such as *Arctica islandica* (#21) because of the effect of suction dredging and, to a lesser extent, predator attacks. A similar process of sediment intrusion, but caused by unsuccessful predator attacks, has been described for *Lutraria lutraria*, *L. magna*, *Panopea glycymeris*, *Ensis siliqua* and *Solen vagina* (#4) and *Tagelus plebeius* (#15). The frequency of repaired shell damage in scallops from different beds analyzed in this study showed a higher percentage in TangoB bed (32%) than Uruguay and Reclutas (12%), with the lower value in Beagle bed (9%). The commercial fishery intensity in the 4 beds showed the higher intensity in TangoB, next Reclutas (with only a minor research disturbance in the area) and Uruguay bed (negligible disturbance) with null fishing intensity (no disturbance) in the Beagle bed. The frequency of damaged scallop observed in those beds-areas not targeted by the fishery (Uruguay, EFA in Reclutas and Beagle bed), assuming not anthropogenic disturbance by fishing, could be related to all natural causes of disturbances that produce injury to be repaired (i.e., predation). The difference between the 32% of scallop damaged in Tango B with values on none (or negligible) disturbed beds could be attributed to the fishery impact or disturbance in the bottom. A positive correlation between the incidence of scars in the shells of *Arctica islandica* (#21), *Ensis siliqua* (#6) and *E. arcuatus* (#19) and the intensity of commercially dredged was observed. If we consider that scallops make a major energetic investment in repairing their shells, which is likely to affect their growth rate at the expense of adductor muscles mass, then the fishery may be having some non-lethal effects on the Patagonian scallop populations. Given the importance in some beds, this potential effect needs to be studied.

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**MORPHOMETRIC VARIATIONS AND INTERNAL SHELL GROWTH PATTERNS OF THE PATAGONIAN SCALLOP *ZYGOCHELAMYS PATAGONICA* ACROSS THEIR SW ATLANTIC DISTRIBUTION RANGE.** Betina Lomovasky,<sup>1</sup> Mario Lasta,<sup>2</sup> Macarena Valiñas,<sup>1</sup> Martín Bruschetti,<sup>1</sup> Silvana Campodónico<sup>2</sup> and Oscar Iribarne.<sup>1</sup> <sup>1</sup>Laboratorio de Ecología, Facultad Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, Mar del Plata, Argentina; <sup>2</sup>INIDEP, Mar del Plata, Argentina

*Zygoclamys patagonica* is widely represented (depth range between 40–200 m; #6, 9, 13, 20) in the extreme of South America (reaching 42°S in the Pacific to Tierra del Fuego (55°S) and 35°50'S in the Atlantic), being an important economic resource in the Atlantic. In the Pacific, main grounds occur in relatively shallow waters (#1) whereas in the Atlantic they are located along the 100 m (#12). Growth and age of *Z. patagonica* were determined from surface external rings (#18, 7) and chondrophores (#20, 19, 5). However, more precise studies are needed to identify internal growth bands, such as acetate peels technique (#16), to validate the external growth rings avoiding error in estimating individual age. Growth, production and physiological activity of bivalves can be affected by environmental conditions (e.g., temperature, salinity, substrate, food-availability; #3, 8, 10, 11). However, density-dependence (#21, 8, 9) and biological interactions could also have an important effect. Individuals from different beds along the Atlantic may have different growth pattern, maximum age and morphometric relationships. We evaluate this prediction comparing different beds along a latitudinal gradient in the south-western Atlantic. Samplings of scallop (24 tows; n = 2,047) were performed in 4 beds (Uruguay: 36°17'S to 53°49'W; Reclutas:

39° 20'S to 56° W and 39° 30'S to 55° 52'W; TangoB: 42° 30'S to 59° 05'W and Beagle: 55° 10'S to 66° 05'W) between January 2000 and October 2004. We measured shell height (H; umbo-ventral margin), length (L; anterior-posterior axis) and width (W; precision  $\pm 0.1$  mm), and total mass without epibionts (TM), shell mass (SM), shell free wet mass (SFWM), gonadal mass (GM), adductor muscle mass (AMM) and epibionts mass (EM; precision  $\pm 0.01$ g). Morphometric relationships were determined and log-log transformations were applied when necessary and compared with ANCOVA between beds. Unplanned comparisons (Tukey multiple comparison, TMC) were made when significant differences were found (#22, 17). For growth and age analysis sub-samples were taken ( $n = 605$ ). Growth pattern observation was inferred from internal shell growth bands using acetate peels technique to allow microscopic examination (#16). The size-mass relationship between TM, SM, SFWM, AMM, GM and EM (dependent variables) and H (independent variable) was exponential in each bed and there was a linear relationship between L and W with H ( $P < 0.01$ ) for all beds. However, the slope of L-H relationship differed between beds ( $P < 0.001$ ); Uruguay and Reclutas beds were similar and differed of TangoB and Beagle (TMC,  $P < 0.05$ ). Similar results were observed for W-H and  $\log(\text{TM})$ - $\log(H)$  relationships. For  $\log(SM)$  and  $\log(SFWM)$  with  $\log(H)$ , the slopes differed between beds ( $P < 0.001$ ). All beds differed each other (TMC,  $P < 0.05$ ). There were no differences for the relationship  $\log(\text{EM})$ - $\log(H)$  between beds (ANCOVA:  $F = 0.71$ ;  $P = 0.5446$ ). There was an increase in all variables following a latitudinal gradient N to S. Under reflecting light, acetate-peels from the polished shell cuts showed a pattern of alternating broad opaque and narrow translucent growth bands. Growth pattern and maximum age differ between beds. There was a similar pattern of internal bands between Uruguay and Reclutas (maximum age of 13 and 14 years old respectively). Internal growth bands in the umbo were clearly observed. It was possible to follow the translucent growth bands all the way along a cross section of the shell to the point where they cross the outer shell layer to form a ring on the exterior shell surface. The 2–3 first bands, difficult to observe along the shell section, showed a different pattern from other growths bands. Each growth break (i.e., an external ring) of the recent bands was formed with a group of internal growth bands (cluster shape). Thus, these clusters were formed by a growth rate slowed down during some periods within a year but not by a total interruption in growth. At the last portion of the valve (recent bands), the internal growth bands were closer to the shell edge and were proximate each other (individuals  $>50$  mm H), clearly visible in acetate peels but difficult to identify on the exterior shell surface. Internal growth bands in the umbo and along the cross section were similar in TangoB but it was difficult to observe the point where the translucent bands cross the outer shell layer to form an exterior ring. The cluster shapes of these exits were less pronounced. Maximum age was 20 years old. Beagle bed showed a higher shell thickness along the cross shell section, as was refluxed in a higher shell mass (see

earlier), where was possible to see all the internal growth bands. The point where the translucent growth bands form an exterior ring did not have a cluster shape; they were a simple translucent growth band. Thus, a total interruption of growth seems to be the cause of this external ring. Maximum age was 21 years old. Acetate-peels technique allows us to obtain new information on the maximum age across the latitudinal gradient. The presence of translucent growth bands indicate periods of very slow or even halted shell growth, possibly caused by low metabolic rates related to a lack of food (#4, 3, 11) or by a diversion of metabolic products into gamete production (#2, 15, 14). The influence of 1°C to 2°C annual variation in temperature in the studied areas seems to be negligible to explain variations detected by our study. However, other factors such as density-dependence, different biological interactions and food availability, could explain the differences found between populations inhabiting different latitudes.

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# THE EFFECT OF PHOTOPERIOD ON THE CONDITIONING OF THE BLACK SCALLOP, *CHILAMYS VARIA*.

**1—GONADAL DEVELOPMENT.** A. Louro,<sup>1</sup> J. P. De la Roche,<sup>1</sup> J. L. Sánchez,<sup>2</sup> A. Silva,<sup>2</sup> P. Martínez,<sup>2</sup> M. L. Pérez-Parallé,<sup>2</sup> I. Martínez<sup>2</sup> and G. Román.<sup>1</sup> <sup>1</sup>Instituto Español de Oceanografía, Centro Oceanográfico de A Coruña, PO Box 130, 15080 A Coruña, Spain; <sup>2</sup>Universidad de Santiago de Compostela, Instituto de Acuicultura, Departamento de Bioquímica y Biología molecular, 15782 Santiago de Compostela, Spain.

Despite different studies having been carried out on the subject, the effect of photoperiod on gametogenesis in bivalves is still not clear. Reproduction was stimulated in *Placopecten magellanicus* (Couturier & Aiken 1989) and *Pecten maximus* (Devauchelle & Mignant 1991, Paulet & Boucher 1991) by increasing the length of photoperiod. Saout et al (1999) found that a simultaneous increase in temperature and length of photoperiod enhanced gonad growth in *P. maximus* when food was not limiting, although they did not determine which of the two factors was the most important. Silva et al. (2002) described acceleration of gonad development of *Ruditapes decussata* in winter, when spring photoperiod conditions were maintained. In this study, comparison is made of gonad development in black scallops subjected to two different day length regimes. Scallops (30 months old) grown by suspended raft culture on the Galician coast were collected in October and November during the gonad-resting period. (De la Roche et al. 2005). Two experiments were carried out; in experiment 1, carried out between November 8, 2002 and 22 February 2003, 264 scallops were used; the mean temperature was  $17.4 \pm 1.5^\circ\text{C}$ . In experiment 2, carried out between 10 October 2003 and 5 January 2004, 280 scallops were used; the mean temperature was  $15.0 \pm 1.1^\circ\text{C}$ . A mixture of *Skeletonema costatum*, *Isochrysis aff. galbana*, *Pavlova lutheri*

and *Rhodomonas salina* cells (mean  $2.5 \times 10^{11}$  cells  $\text{day}^{-1}$ ) was added to the tanks ( $180 \times 50 \times 30$  cm) in which the scallops were maintained. In each experiment, the scallops were divided in two groups of equal number, and subjected to a day length of 16 h (LP) or a day length of 8 h (SP). A total of 15 individuals were dissected to determine initial conditions. Samples of 15 scallops were periodically removed from each experimental tank, the different organs dissected and the dry weight recorded. A predictive regression between height and dry weight of the organs was calculated and the mean values for a standard scallop of 50-mm height was estimated. There were clear differences in gonad development in scallops maintained under the different day-length regimes. In experiment 1, under LP conditions, gonads showed growth at 15 days, and maximum values (mean 0.23 g) were reached after 60 days, followed by spawning. Scallops maintained in SP conditions showed slower gonad development, the maximum values (mean 0.16 g) were reached after 82 days, followed by spawning. In experiment 2, very fast gonad growth was recorded under LP conditions, reaching a peak value (0.25 g) followed by spawning, after 40 days. The subsequent decrease in gonad size was followed by a rapid recovery and a new peak value (mean 0.35 g) was recorded 1 month later and was followed by a second spawning. Scallops maintained under SP conditions did not develop gonads between October and December. In general, the digestive gland showed a slight increase in weight parallel to that of the gonad, probably because as gonad grows it envelops the gland, and the weight of the digestive gland included a small amount of gonad. The weight of muscle tended to decrease throughout the conditioning period. Variations in the dry weight of the remaining tissues were not observed. Under conditions of spring day length (LP), at moderate temperatures (experiment 2,  $15.0 \pm 1.1^\circ\text{C}$ ) and

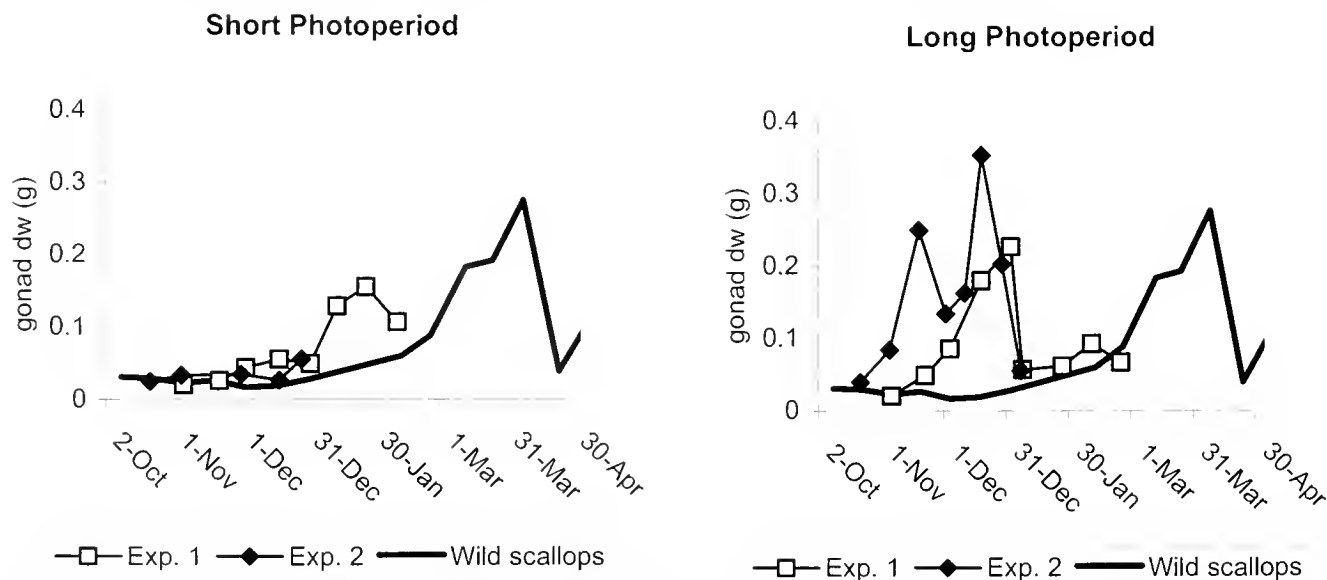


Figure 1. Changes in gonad dry weight in wild scallops and in scallops held under two photoperiods and temperatures. Values are standardized for a shell height of 50 mm.

with abundant food, in approximately 3 months (October to December)

The scallops underwent the complete bimodal breeding cycle previously recorded under natural conditions at sea (February to June, De la Roche et al. 2005), whereas under conditions of winter day length (SP) gonad growth failed to start. At higher temperatures (experiment 1,  $17.4 \pm 1.5^\circ\text{C}$ ) gonad growth took place under both conditions of day length. In the latter case, the gonad development under LP conditions was slower than in scallops maintained at  $15^\circ\text{C}$ . However, at  $17.4^\circ\text{C}$  gonad growth took place even in scallops maintained under SP conditions.

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**DETERMINING THE OPTIMAL SCALLOP SPAT LENGTH AND TRANSPORT CONDITIONS FOR *PECTEN NOVAEZELANDIAE* DURING THE SEEDING ENHANCEMENT PROGRAM IN NELSON, NEW ZEALAND.** Rebecca L. Lyon<sup>1</sup> and Islay D. Marsden.<sup>2</sup> <sup>1</sup>Ministry of Fisheries, Aquaculture Team, Private Bag 14, Nelson, New Zealand; <sup>2</sup>School of Biological Sciences, University of Canterbury, Private Bag 4800, Christchurch, New Zealand.

Scallop meat weight landings in the last 5 years peaked at 716 tons in the 2001/2002 season and was worth an estimated \$26 million dollars. The success of the scallop industry relies heavily on a spat enhancement programme run by the Challenger Scallop Enhancement Company Limited. Mesh spat collectors are set during November or December and harvested around March (primary spat). Fall off from the primary spat collectors results in high densities of juvenile scallops on the seafloor, which are harvested as secondary spat. The aim of this research is to determine which length scallop spat was the most robust and therefore most likely to survive the harvesting and relocation processes. Behavioral experiments were conducted on primary spat from 9 different length groups (8–10 mm, 11–12 mm, 13–15 mm, 16–17 mm, 18–20 mm, 21–22 mm, 23–25 mm, 26–27 mm, 28–30 mm) collected during the 2002 primary harvest. When presented with a starfish predator (*Coscinasterias calamaria*), 10 mm spat had a significantly slower reaction time ( $8.30 \pm 0.16$  sec) than other length groups and 17 mm length group had the fastest reaction time ( $2.55 \pm 0.32$  sec). Small spat had significantly fewer adductions in their first swimming

bout than large spat (12 mm =  $8.44 \pm 0.61$ , 27 mm =  $10.90 \pm 0.49$ ). Also, small spat exhibited fewer overall adductions than large spat, (10 mm =  $2.24 \pm 1.58$ , 27 mm =  $40.52 \pm 1.20$ ). Aerial exposure experiments conducted on 3 length groups (20–35 mm, 36–50 mm, 51–65 mm) indicated that scallops could survive up to 8 hours transport at temperatures  $<20^\circ\text{C}$ . When scallops were acclimated to  $18^\circ\text{C}$ , mortality was approximately 15%. However, over 2 hours exposure at a temperature of  $30^\circ\text{C}$  resulted in 60% to 100% mortality. This study suggests that scallop spat  $>20$  mm from the primary harvest are the most robust. The secondary harvest should target larger juvenile scallops ( $>27$  mm) when transporting long distances ( $>8$  hours).

**TOLERANCE, RESISTANCE AND OPTIMUM VALUES OF TEMPERATURE AND SALINITY, IN THE GIANT LION'S PAW SCALLOP (*NODIPECTEN SUBNODOSUS*).** Alfonso N. Maeda-Martínez, Teresa Sicard-González, Lucelly M. Roldán-Carrillo and Flavio González-Estrada. Centro de Investigaciones Biológicas del Noroeste (CIBNOR), Mar Bermejo 195, Col. Playa Palo de Santa Rita, La Paz, B.C.S. 23090, Mexico.

Lower and upper tolerance and resistance and optimum values for growth under different temperature and salinity ranges were studied in juveniles of the giant lion's paw scallop *Nodipecten subnodosus*. Tolerances were measured with the median lethal dose method ( $\text{LD}_{50}$ –96 h), exposing specimens acclimated initially at 15, 18, 22, 26, or  $30^\circ\text{C}$  and 37 psu, to 13 temperatures (1, 3, 5, 10, 15, 26, 28, 30, 31, 32, 33, 34, or  $35^\circ\text{C}$ ). Halotolerance was determined by subjecting acclimated scallops at  $22^\circ\text{C}$  and 37 psu (control conditions) to 9 salinities (15, 20, 25, 30, 37, 42, 47, 50, or 60 psu). Resistance to both variables were studied using a gradual change, decreasing or increasing the temperature  $1^\circ\text{C}/\text{day}$  or salinity 3 psu every 3 days from the acclimation temperatures and salinities, until mortality of the test specimens occurred. Optimum values were defined by measuring the scope for growth and by somatic shell and biomass growth determinations at different temperatures (15, 18, 22, 26 and  $30^\circ\text{C}$ ) and salinities (25, 30, 37, 43, and 47 psu). Results showed that the lower and upper temperature tolerance at 96 h varied from  $5.1^\circ\text{C}$  to  $12.5^\circ\text{C}$  and  $27.8^\circ\text{C}$  to  $32.9^\circ\text{C}$  at  $15^\circ\text{C}$  and  $30^\circ\text{C}$  acclimation, respectively. Salt tolerance varied from 24.2 to 53.8 psu. The lower temperature resistance was not achieved at the lowest temperature tested ( $3^\circ\text{C}$ ) and the upper resistance value was  $32^\circ\text{C}$ , independent of the acclimation temperature. Salt resistance experiments showed no mortality in the 22 to 52 psu range, but abrupt mortality occurred with exposure beyond these values. Scope for growth and somatic growth experiments indicated that the optimum temperature and salinity were  $22^\circ\text{C}$  and 37 psu. The application of these results to hatchery operations and site selection for growout of giant lion's paw scallop is discussed.

**THE EFFECT OF PHOTOPERIOD ON THE CONDITIONING OF THE BLACK SCALLOP, *CHLAMYS VARIA* II—HISTOLOGICAL STUDY.** I. Martínez,<sup>1</sup> A. J. Pazos,<sup>1</sup> A. Louro,<sup>2</sup> J. P. de la Roche,<sup>2</sup> G. Román,<sup>2</sup> M. Abad<sup>1</sup> and J. L. Sanchez.<sup>1</sup> <sup>1</sup>Instituto de Acuicultura, Departamento de Bioquímica y Biología Molecular, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, Spain; <sup>2</sup>Instituto Español de Oceanografía, Centro Oceanográfico de A Coruña, PO Box 130, 15080 A Coruña, Spain.

The aim of this study is to achieve some progress in the optimization of the conditioning of the black scallop, *Chlamys varia* through suitable manipulation of photoperiod. Several authors have reported that daylight has an important influence on the gametogenesis in marine organism (Pearse et al. 1986, Devauchelle & Mingant 1991, Paulet & Boucher 1991, Saout et al. 1999, Chavez-Villalba et al. 2002). However, results of previous studies on the influence of photoperiod on gametogenic development are often contradictory (Devauchelle & Mingant 1991), and therefore it is not usually taken into account when conditioning procedures are developed (Utting & Millican 1997). We tested black scallops at two different stages of gametogenesis because we assume that the response will differ according to the initial reproductive stage of the progenitors. Two conditioning experiments were carried out (experiment 1: November to February, experiment 2: March to April). Adult scallops grown by raft culture in Galicia, were held in tanks (180 × 50 × 30 cm) in seawater for the conditioning. The mean temperature was 17.4 ± 1.5°C in experiment 1 and 15.0 ± 1.1°C in the experiment 2. Scallops were fed a mixed diet of *Skeletonema costatum*, *Isochrysis aff. galbana*, *Pavlova lutheri* and *Rhodomonas salina* (2.5 × 10<sup>11</sup> cells · day<sup>-1</sup>). In each experiment, two photoperiods were tested; in three tanks scallops were maintained fewer than 8 h light and 16 h dark (winter conditions), whereas in other three tanks were maintained under 16 h light and 8 h dark (spring conditions). Every 2 weeks 15 scallops were sampled from each experimental tank and the gonad dissected out. Gonad tissue was fixed in Bouin (5 h) and the tissues were dehydrated in series of alcohols, and embedded in wax. Histological sections (3-μ thick) of each gonad were stained by the Wheatley trichrome method (1951). Specimens were examined microscopically for evidence of gametogenesis. Experiment 1: the scallops maintained under the 16 h light regime showed signs of gametogenesis development after only 15 days of conditioning, whereas scallops maintained under the 8 h light regime remained in resting state after 30 days of conditioning and gonad development was not observed until after 45 days of conditioning, by which time the group of scallops under the 16 h light regime had already spawned. Experiment 2: In scallops subjected to the 8 h light regime there was a certain degree of regression, whereas scallops subjected to the 16 h light regime achieved spontaneous spawning during the first month of conditioning. These results are consistent with those of Saout et al. (1999) who reported that photoperiod is undoubtedly the most important factor involved in the synchronization of

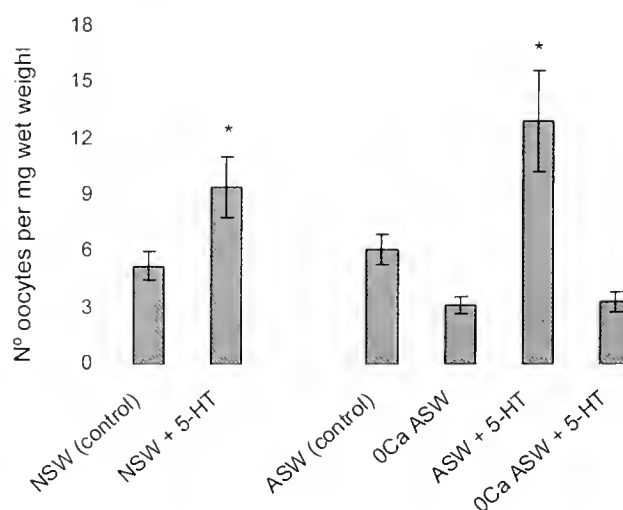
reproduction on pectinids, whereas temperature and nutrition are more important in terms of the speed of the gametogenesis once the process has been established in the animals.

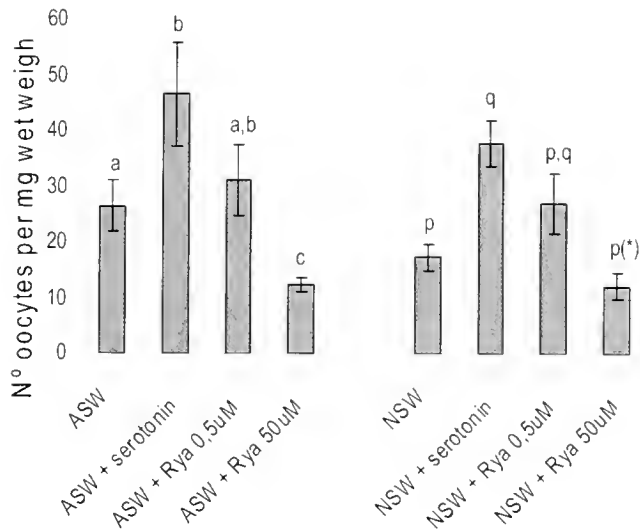
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**CALCIUM IS AN ESSENTIAL ION FOR THE SPAWNING AND RESUMPTION OF MEIOSIS OF THE SCALLOP *ARGOPECTEN PURPURATUS*.** Gloria Martínez, Miguel Angel Pérez and Livia Mettifogo. Facultad de Ciencias del Mar, Universidad Católica del Norte, Larrondo 1281, Coquimbo, Chile.

For some years our research has focused on understanding the control of spawning in the scallop *Argopecten purpuratus*. We have investigated the dynamics of some monoamines and second messengers during this process. Lately, we have shown an increase of inositol 1-, 4-, 5-trisphosphate (IP<sub>3</sub>) associated with spawning (Martínez & Cisterna 2004). This messenger binds to the IP<sub>3</sub> receptor, a Ca<sup>2+</sup> channel in the reticulum endoplasmic membrane and thereby releases Ca<sup>2+</sup> into the cytoplasm. This finding led us to think that Ca<sup>2+</sup> is involved in the spawning process. Because smooth muscle contraction is a Ca<sup>2+</sup>-sensitive process, the IP<sub>3</sub> rise may be a required step for increasing intracellular Ca<sup>2+</sup>, which is necessary for the contraction of gonadal muscular tissue and the expelling of gametes. Whereas meiosis in this species is reinitiated





before female gametes are released to the environment, we postulated that  $\text{Ca}^{2+}$  could also be necessary for meiosis resumption. The aim of the present work is to investigate “*in vitro*” the need of calcium ion for the release of gametes and for meiosis resumption in *A. purpuratus*. Female portions of ripe *A. purpuratus* gonads were cut into small pieces of 30–50 mg wet weight and washed with filtered seawater. Pieces originating from the same individual were incubated for 90 minutes in separate 2 mL glass vials containing one of the following solutions: Artificial seawater (ASW), ASW without  $\text{Ca}^{2+}$  (0Ca ASW), ASW plus  $10^{-5}$  M serotonin (5-HT), 0Ca ASW + 5-HT, filtered natural seawater (NSW), NSW +  $10^{-5}$  M 5-HT, NSW + 0.25  $\mu\text{M}$  Ryanodine (Rya), NSW + 0.50  $\mu\text{M}$  Rya, NSW + 50  $\mu\text{M}$  Rya, NSW + 10 mM caffeine and NSW + 20 mM caffeine. After incubation, the liberated oocytes were fixed in Carnoy solution for their later counting under light microscope. Different experiments were run to assay the different treatments but each time, the experimental and control solutions were assayed on tissue coming from the same individuals. All the

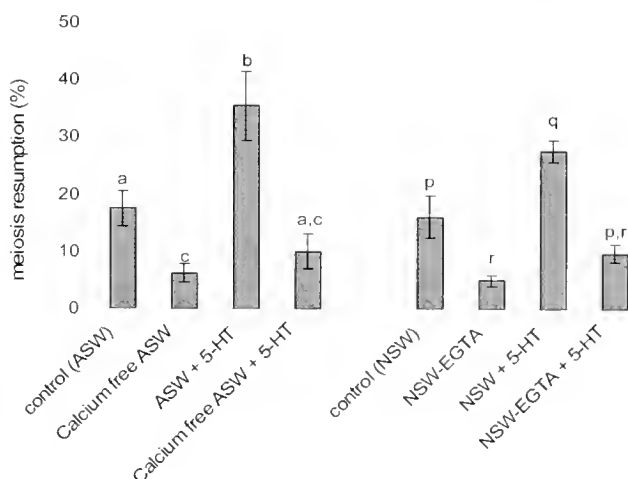
experimental treatments were replicated eight times, each with a different scallop. Data obtained were subjected to 1-way analysis of variance followed by a Tukey test in order to compare the differences ( $P < 0.05$ ). Gross transverse sections of about 2 mm thickness were obtained from female gonadal portions and were placed in glass dishes containing 5 mL of some of one of the following solutions: NSW, NSW + 50 mM EGTA, NSW +  $10^{-5}$  M 5-HT, NSW + 50 mM EGTA +  $10^{-5}$  M 5-HT, ASW, 0Ca ASW, 0Ca ASW + 5-HT. After 60 minutes, each tissue sample was rinsed with NSW or ASW and superficial scrapes were obtained with a surgical scalpel and were fixed with Carnoy solution. The total number of oocytes, and the number that showed meiosis resumption (visualized by germinal vesicle breakdown, GVBD) were counted under a microscope to calculate GVBD percent. Percentages, subjected to arc-sin transformation were analyzed by 1-way ANOVA. The release of oocytes from gonadal pieces was inhibited when  $\text{Ca}^{2+}$  was absent from the incubation solution and the usual stimulatory effect of serotonin was blocked. Ryanodine is an alkaloid known to bind  $\text{Ca}^{2+}$  channels (ryanodine receptors, RyR) in the sarcoplasmic reticulum (SR) and is involved in the release of this ion from intracellular stores. This alkaloid inhibited the release of oocytes at 50  $\mu\text{M}$  in normal and artificial seawater. Low concentrations (nanomolar range) of Rya render the membrane of SR leaky to  $\text{Ca}^{2+}$ , whereas higher values ( $\mu\text{M}$ olar range) prevent  $\text{Ca}^{2+}$  release (Williams & Tanna 2004). This result suggests that RyR are involved in the process of oocyte release and that  $\text{Ca}^{2+}$  for the spawning is moving from SR stores. To confirm this we used caffeine, a methylxanthine known to increase  $\text{Ca}^{2+}$  conductance by RyR (Zucchi & Ronca-Testoni 1997). The release of oocytes was significantly increased when 10 and 20 mM caffeine were used in the incubation medium. Meiosis resumption was blocked in the absence of calcium ion, effect that was detected either in ASW without  $\text{Ca}^{2+}$  or in NSW plus the calcium quelator EGTA. Calcium ion must be present in the medium for meiosis resumption and release of oocytes from gonad of *A. purpuratus*.

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**COMMERCIAL SCALLOP CULTURE TRIALS AT THE PINNACE CHANNEL AQUACULTURE FISHERIES RESERVE, IN SOUTHERN AUSTRALIA.** John A. Mercer,<sup>1</sup> Neil J. Hickman<sup>2</sup> and Richard J. Gasior.<sup>2</sup> <sup>1</sup>Fisheries Victoria, Department of Primary Industries, PO Box 114 Queenscliff, Victoria 3225, Australia; <sup>2</sup>Primary Industries Research Victoria, Department of Primary Industries, PO Box 114 Queenscliff, Victoria 3225, Australia.

Fisheries Victoria facilitated a scallop growing trial at the new 1,000 ha Pinnacle Channel Aquaculture Fisheries Reserve (PCAFR) in southern Port Phillip Bay, Victoria. The aim of the



trial is to evaluate the Reserves' suitability for scallop culture through a series of commercial trials undertaken by prospective scallop farmers. An industry consortium monitored scallop larvae abundance in the water at the PCAFR for 2 years, in order to determine if it were possible to catch commercial quantities of scallops in "spat-bags." Scallops that were caught at the site were used for a range of growing trials. The trial reported here grew a crop of scallops on two under-water commercial long-lines that were held 10 m below the sea-surface in water that was approximately 20 m deep. The seed scallops collected from spat bags in April 2003 were used to evaluate a range of culture nets and containers. The trials involved PIRVic undertaking a trial using a "standard" culture method and industry conducting a range of trials using various culture systems. These trials were run concurrently on the sub surface long-lines. Primary Industries Research Victoria (PIRVic) was commissioned to measure growth rate and meat condition of scallops grown by a "standard" method that used pearl nets for the early nursery stage and box nets for the final grow-out. PIRVic also assisted industry in assessing other methods that were used, including a variety of oyster containers and shellfish growing nets. When using the "standard" growing method, seed scallops that averaged 22.7 mm in length at the start, grew to 79.1 mm with negligible mortality in 18 months. The scallops grew throughout the whole year with slower rates over the colder winter months. Scallop meat condition was variable and ranged from good to poor. An assessment of commercial production was made after 18 months. Only one of the commercial methods investigated performed as well as the scallops that were grown in pearl and box nets and measured regularly by the "standard" growing method. This method was growing scallops in 4.5 mm pearl nets for 9 months then at a low density in large round lantern nets for the final 9 months. All other treatments tested did not perform as well as the "standard" method. It was concluded that many of the physical attributes of the site make the PCAFR a good location for commercial scallop aquaculture. The establishment of the PCAFR by the Victorian Government provides an opportunity for prospective marine farmers to develop a new shellfish aquaculture industry.

**MAKING IT WORK—SCALLOP CULTURE AND MANAGEMENT IN NEW ZEALAND.** Russell Mincher, Challenger Scallop Enhancement Ltd., P.O. Box 175, Nelson, New Zealand.

Commercial success in fisheries management seldom has a great deal to do with managing the fish. Fish reproduce, grow and die as they have done without human intervention since time immemorial. The fishery manager is concerned with managing people and their behavior so that the fish can get on with the business of growing to harvest condition. That premise holds true for all exploited fisheries, including scallop fisheries whether they are farmed, ranched, enhanced or truly wild. Managing an enhanced scallop fishery to generate a commercial outcome is a

business. It is the business of using the resources available to the organization to influence the productivity, security and profitability of harvests over the long term. The Challenger Scallop Enhancement Company of New Zealand (Challenger) is in that business. Information needs and scientific inputs required by fishery managers in an enhanced fishery change as the fishery matures. Having settled how best to intervene in the reproductive cycle of the fishery to successfully enhance, the manager's focus shifts to improving harvests, improving operational efficiency and resolving threats to the fishery or program. Russell's address describes some of the challenges previously overcome and others currently faced by Challenger as it manages a commercialised scallop enhancement program. Those challenges include:

- Securing on-going funding for the program
- Managing the interface with recreational and customary scallop fishers
- Improving spat survival rates while reducing the costs of enhancement
- Managing harvester behavior
- Protecting the fishery from conflicts with marine farming proposals

**SCALLOP (*PLACOPECTEN MAGELLANICUS*) SEA SAMPLING PROGRAM AS A STOCK STATUS INDICATOR.** Monique Niles and Leslie-Anne Davidson, Aquaculture Division, Department of Fisheries and Oceans, Moncton, New Brunswick, E1C 9B6, Canada.

The sea scallop (*Placopecten magellanicus*) fishing grounds in the southern Gulf of St. Lawrence are divided into four Areas. The scallop fishery is managed by imposing seasons, meat count limits, gear size limits and restrictions and logbook reporting. Over the last 4 years, the landings have hovered above 100 mt of meat, the lowest recorded since 1968 when peak values attained 900 mt. Since implementing logbook reporting in 1999, it has become possible to map fishing positions and effort. In 2001, a sea sampling program was put in place in collaboration with the fishing industry to better understand the status and biology of the scallop beds. As a result, recommendations have been made to fishermen and fisheries managers concerning meat count limits and ring size increases. Meat count regulations have decreased from 52 meats/500 g to 44 meats/500 g in one Area, while the ring size has increased from 3 inches to 3¼ inches in all Areas. Also, it has been demonstrated to fishermen that returning scallops to sea that are smaller than 80 mm will not jeopardize their current landings. By adopting these recommendations, it is thought that over time, fishermen could increase their landings and maximize meat yields by targeting scallops larger than 100 mm. Trends in the meat weights of 90 mm scallops have been evaluated against environmental parameters. The sea sampling program has proven to be an inex-



pensive but useful tool in better understanding the scallop stocks, fishery and biology and in giving science-based advice.

**AUSTRALIAN SAUCER SCALLOPS—SEA RANCHING GENETICS.** Elizabeth O'Brien,<sup>1</sup> Jason Bartlett,<sup>1</sup> Bryony Dixon<sup>1</sup> and Peter Duncan.<sup>2</sup> <sup>1</sup>Department of Primary Industries and Fisheries, Profitable Aquaculture Systems, Bribie Island Aquaculture Research Centre, PO Box 2066, Woorim, QLD 4507, Australia; <sup>2</sup>Faculty of Science, University of the Sunshine Coast, Maroochydore DC, QLD 4558, Australia

The saucer scallop, *Amusium balloti*, is distributed along the Western and Eastern coast of Australia and supports a fishery in both states. To stabilize the annual catch rates, private companies from both states are investing in sea ranching or reseeded operations. Because of biological constraints on wild harvest of spat, the enhancement operations are reliant on production of seed from hatcheries. The mass release of scallop juveniles is expected to have an immediate effect on stock abundance but it also has the potential to alter the genetic structure of the wild saucer scallop populations. To minimize the environmental impacts of reseeded, genetic resource management is recommended, and both companies are supportive of understanding the genetic structure of the native populations. To facilitate this, the genetic population structure of saucer scallops along the Western and Eastern Australian coastline have been analyzed using eight microsatellite loci and the results will be presented during this presentation. Previous allozyme work compared the Queensland and Western Australian forms of *A. balloti*, and results indicated that they might be classed as different species (Dredge et al. in prep.). We have used 16S and 12S rRNA sequence fragments to verify these findings and present the results here. Finally we will present a suggested genetic management strategy for Australian saucer scallop ranching, based on these findings.

**THE EFFECT OF TRANSPORTATION ON THE PHYSIOLOGICAL CONDITION AND FREE AMINO ACIDS COMPOSITION OF *NODIPECTEN SUBNODOSUS* ADDUCTOR MUSCLE.** V. M. Ocaño-Higuera,<sup>1</sup> A. N. Maeda-Martínez,<sup>2</sup> M. E. Lugo-Sánchez<sup>3</sup> and R. Pacheco-Aguilar.<sup>3</sup> <sup>1</sup>Universidad de Sonora, Departamento de Ciencias Químico Biológicas, Boulevard Luis Encinas y Rosales, Col. Centro, C.P. 83000; <sup>2</sup>Centro de Investigaciones Biológicas del Noroeste, S.C. Mar Bermejo No. 195, Col. Playa Palo de Santa Rita, Apdo. Postal 128, La Paz, BCS 23090, México; <sup>3</sup>Centro de Investigación en Alimentación y Desarrollo A.C. Carretera a la Victoria Km 0.6, CP. 83000, Hermosillo, Son. Apdo. Postal 1735.

The lion's paw scallop, *Nodipecten subnodosus* (Sowerby, 1835) is the largest pectinid in the Baja California Peninsula, Mexico. This scallop is distributed from Ojo de Liebre lagoon, Baja California Sur México, to Peru. At the present time, some

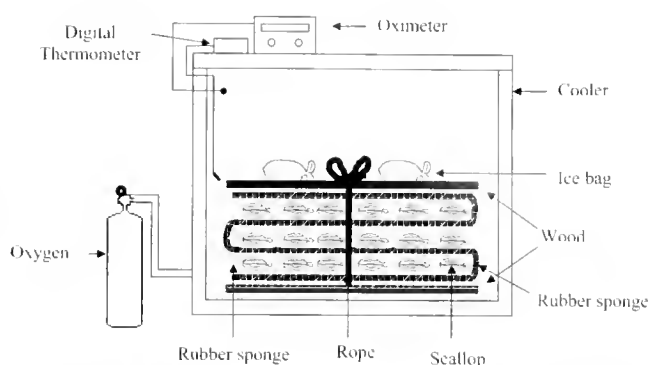


Figure 1. Device used for shipping of lion's paw scallop *Nodipecten subnodosus*.

aspects on culture, reproductive cycle, growth, gametogenesis, postmortem changes and quality of the adductor muscle has been studied in *Nodipecten subnodosus*. However, reports of the transportation effect on the physiology and free amino acids composition in the adductor muscle is very scarce. Therefore the overall objective of this study is to evaluate the effect of transport on the physiology of this scallop, using the changes in adenylic energetic charge, glycogen content, muscle yield index and free amino acids composition as indicators of stress. Scallops *Nodipecten subnodosus* of 6–7 cm shell height were used in the study. A sample of 2,630 individuals was collected and transported from the Guerrero Negro lagoon to the laboratory at the Universidad Autónoma de Baja California Sur, Mexico. The elapsed time for transportation was 11 h. Features of the original shipping device designed by Maeda-Martínez et al. (2000) for scallop seed was substantially modified and shown in Figure 1. Firstly, the organisms were placed on a wood base (100 cm in diameter) and sponge rubber over which, alternate beds of organisms—sponge rubber—organisms were placed. The sponges were soaked with seawater during the packing. Afterwards, a base of wood was collocated on the top, which served as a lid. A rope was used to secure the sandwich of organisms, which was placed inside a 1m<sup>3</sup> cooler

TABLE 1.

Average weights (N = 6) of the adductor muscle, gonad, total tissue and whole organism (shell + tissues) from lagoon and post-transport.

	Adductor Muscle (g)	Gonad (g)	Total tissue Weight (g)	Total Weight (g)
Scallops from lagoon	7.0 ± 1.8 <sup>a</sup>	2.5 ± 0.7 <sup>a</sup>	21.6 ± 4.5 <sup>a</sup>	61.0 ± 12.2 <sup>a</sup>
Scallops post-transport	7.2 ± 1.5 <sup>a</sup>	2.0 ± 1.3 <sup>a</sup>	17.9 ± 3.2 <sup>a</sup>	60.5 ± 9.8 <sup>a</sup>

Data represent the mean and standard deviation of n = 6

Different letter in the same column indicates significant differences (P < 0.05)

TABLE 2.

Means  $\pm$  SD of total carbohydrates, glycogen, ATP, ADP, AMP and calculated AEC levels in the adductor muscle of lion's paw scallop (*Nodipecten subnodosus*) before and after transport.

	Total Carbohydrates (mg/g)*	Glycogen* (mg/g)	ATP ( $\mu$ M/g of Muscle)	ADP ( $\mu$ M/g of Muscle)	AMP ( $\mu$ M/g of Muscle)	AEC	Muscle Yield Index (%) <sup>2</sup>
Scallops from lagoon	243.4 $\pm$ 25.6 <sup>a</sup> (203.6–268.5)	230.1 $\pm$ 24.7 <sup>a</sup> (201.4–253.0)	7.4 $\pm$ 2.2 <sup>a</sup> (4.9–10.6)	2.3 $\pm$ 1.0 <sup>a</sup> (1.5–4.1)	0.6 $\pm$ 0.4 <sup>a</sup> (0.17–1.3)	0.8 $\pm$ 0.1 <sup>a</sup> (0.70–0.91)	32.2 $\pm$ 5.7 <sup>a</sup>
Scallops post-transport	15.4 $\pm$ 7.1 <sup>b</sup> (8.0–24.5)	12.6 $\pm$ 6.6 (6.1–22.4)	3.7 $\pm$ 1.8 <sup>b</sup> (0.8–5.6)	3.7 $\pm$ 1.1 <sup>b</sup> (2.6–5.54)	3.9 $\pm$ 2.0 <sup>b</sup> (1.64–5.89)	0.5 $\pm$ 0.2 <sup>b</sup> (0.2–0.7)	40.4 $\pm$ 5.8 <sup>b</sup>

The values are the mean  $\pm$  SD of  $n = 6$ .

Different letter in the same column indicate significant differences ( $P < 0.05$ )

adapted with an oximeter and a thermometer. Ice bags were placed inside the cooler, then sealed with a rope and filled with O<sub>2</sub> organisms from the lagoon, before and after shipping, were frozen in liquid nitrogen. Average weights for adductor muscle, gonad total tissue and total weight of the scallop are shown in Table 1. No significant changes ( $P > 0.05$ ) were observed between the organisms before and after transportation, however a slight change was observed in the total tissue weight caused by the liberation of water from the mantle, this fact needs to be considered in more detail in further studies. Table 2 shows the average levels of glycogen content, total carbohydrates, ATP, ADP, AMP, adenylic energetic charge and muscle yield index of the adductor muscle of lion's paw scallop from lagoon and post-transport. A significant decreased ( $P < 0.05$ ) in the glycogen content, total carbohydrates,

ATP and adenylic energetic charge was observed after transport, because these compounds are used by the organisms to compensate the stress produced during the transport and maintain the good operation of their organs. On the other hand, the ADP and AMP concentrations as well as the muscle yield index increased significantly ( $P < 0.05$ ) because of breakdown of ATP, which produces the accumulation of ADP and AMP and the liberation of water, respectively. Finally, Table 3 shows the free amino acid composition in the adductor muscle of lion's paw scallop and the effect of transportation on their concentration. Glycine, taurine, arginine and alanine were the most dominant free amino acids and accounted for 74.2% of the total. After the transport, a significant increase ( $P < 0.05$ ) in the concentration of free amino acids (individual and total) was observed. This increase was produced by the dehydration during the transportation, since free amino acids, which are important in maintaining normal physiological and biological functions, are used together with potassium, bicarbonate and phosphate compounds as osmotic effectors in marine bivalves. In conclusion, the method used to transport the organisms was successful due to the high rate of survival (88 %) and the quantity of the transported organisms

TABLE 3.

Free aminoacids—Initial and post-transport composition of free amino acids in the adductor muscle of *Nodipecten subnodosus*.

Amino Acid	(mg/100 g of Muscle) Scallop From Lagoon	Post-transport
Glycine	1454.8 $\pm$ 310.3 <sup>a</sup>	1540.8 $\pm$ 139.0 <sup>a</sup>
Taurine	761.1 $\pm$ 163.8 <sup>a</sup>	764.6 $\pm$ 65.4 <sup>a</sup>
Arginine	337.0 $\pm$ 82.2 <sup>a</sup>	797.6 $\pm$ 94.9 <sup>b</sup>
Alanine	225.1 $\pm$ 81.3 <sup>a</sup>	129.1 $\pm$ 45.0 <sup>b</sup>
Glutamic acid	141.0 $\pm$ 27.1 <sup>a</sup>	138.6 $\pm$ 28.0 <sup>a</sup>
Histidine	110.3 $\pm$ 49.7 <sup>a</sup>	126.4 $\pm$ 57.7 <sup>a</sup>
Aspartic acid	72.1 $\pm$ 25.1 <sup>a</sup>	76.2 $\pm$ 33.7 <sup>a</sup>
Threonine	61.1 $\pm$ 15.5 <sup>a</sup>	201.6 $\pm$ 36.7 <sup>b</sup>
Serine	25.8 $\pm$ 11.4 <sup>a</sup>	127.6 $\pm$ 33.6 <sup>b</sup>
Lysine	25.4 $\pm$ 7.3 <sup>a</sup>	137.6 $\pm$ 50.2 <sup>b</sup>
Valine	23.9 $\pm$ 7.4 <sup>a</sup>	112.9 $\pm$ 36.8 <sup>b</sup>
Phenylalanine	13.3 $\pm$ 3.5 <sup>a</sup>	111.7 $\pm$ 37.5 <sup>b</sup>
Tyrosine	12.8 $\pm$ 4.3 <sup>a</sup>	111.2 $\pm$ 26.8 <sup>b</sup>
Leucine	10.8 $\pm$ 2.8 <sup>a</sup>	137.1 $\pm$ 47.7 <sup>b</sup>
Methionine	8.4 $\pm$ 4.1 <sup>a</sup>	46.2 $\pm$ 16.7 <sup>b</sup>
Isoleucine	8.2 $\pm$ 1.9 <sup>a</sup>	93.9 $\pm$ 51.9 <sup>b</sup>
Total (mg/g of muscle)	3637.7 $\pm$ 919.6	4353.2 $\pm$ 459.1

The values are the mean  $\pm$  SD of  $n = 6$ .

Different letter in the same row indicate significant differences ( $p < 0.05$ )

**GREAT SCALLOP MYOSTATIN—THE ROLE AS A NEGATIVE MUSCLE GROWTH REGULATOR ALSO IN INVERTEBRATES?** H. Pagander,<sup>1</sup> O. Strand,<sup>2</sup> T. Magnesen,<sup>3</sup> I. A. Johnston<sup>4</sup> and O. Andersen.<sup>1</sup> <sup>1</sup>AKVAFORSK, P.O. Box 5010, 1432 As-NLH; <sup>2</sup>Inst. Marine Research, P.O. Box 1870 Nordnes, N-5817 Bergen; <sup>3</sup>SCALPRO AS, 5337 Rong; <sup>4</sup>Gatty Marine Laboratory, University of St. Andrews, Scotland.

Cultivation of scallop has received much attention because of the increasing demand for high quality seafood products at the European market. The highly favorable conditions for growing the great scallop, *Pecten maximus*, along the Norwegian coastline give a unique opportunity to become a leading nation for scallop production. This project is focusing on the environmental and genetic regulation of the larval growth and development of the large adductor muscle, which is the primary product sold. The muscle fiber

anatomy and recruitment in molluscs have been studied for the first time. Water temperatures of 14°C or 18°C at early developmental stages were shown to influence larval growth and survival. Genes encoding contractile proteins and regulatory factors have been partially isolated, including the negative muscle growth regulator myostatin. Great scallop myostatin showed about 50% sequence similarity to fish and mammalian myostatin in the active C-terminal portion. The construction of an EST-library from the scallop adductor muscle is in progress to reveal the full-length cDNA sequences of additional muscle regulatory genes. *In situ* hybridization and immunohistochemistry will then be used to study the sub-cellular location of the gene products during growth and recruitment of the muscle cells.

**ADOPTING AREA-BASED HARVESTING STRATEGIES IN THE TASMANIAN SCALLOP FISHERY.** Rod Pearn and Hilary Revill. Department of Primary Industries, Water and Environment, Tasmania, Australia.

The Tasmanian scallop fishery is primarily based on the harvest of the commercial scallop (*Pecten fumatus*). Scallop fisheries worldwide are notorious for their variability in recruitment. The Tasmanian fishery is no exception to this, and historically, the fishery has been subject to highly variable annual catches and total fishery closures. The fishery has only opened in a total of 6 years in the last decade. The high variability in stock abundance is strongly influenced by environmental factors. In 2003, the fishery adopted a "tight" spatial management strategy with the aim of increasing the likelihood of fishing continuity between years. The strategy is different from the majority of other fisheries in that all areas are closed unless specifically opened. The strategy limits fishing to discrete areas and aims to:

- maximize stock rebuilding in unfished areas
- protect stock in other areas for future access
- limit fishing impacts on under size scallops
- limit fishing impacts on the broader marine environment

Seasonal closures are in force during the period when spawning and larval settlement are likely. The closed season also protects newly settled scallops.

The spatial management system also incorporates 3 levels of closure.

- Permanent closed areas: notably all shark refuge areas are closed to scallop dredge activities.
- Closed Area (class 1) areas may be declared prohibiting all vessels with a scallop dredge or active scallop license being in an area, including transiting the area. This type of closure maximizes the protection of scallops, especially where they are in close proximity to open areas.
- Closed Area (class 2) areas are all other state waters not design-

nated, as open. Scallop vessels can transit these areas, however cannot scallop fish or have any scallop dredge in the water. All vessels participating in the scallop fishery are required to have a vessel monitoring systems (VMS), which allows real time monitoring of vessel positions.

Generally, before any area is considered for opening a survey is conducted either by permit or by scientific assessment. An area may be considered for opening if there are substantial scallop beds the area consisting of less than 20 % undersize scallops.

Previous survey information, past history and anecdotal information maybe used to determine where the survey will be conducted. The Spatial management system where the majority of waters a closed in to fishing, however presents challenges in identifying scallop beds outside open areas. Potentially this either increases research costs or limits information. The need for increased spatial information has been identified as a major challenge by industry and the management agency. Over the last 2 years various forms of industry based surveys have been trialed, with the aim of increasing information about the scallop resource. The need for stock information on a wider geographical scale to determine future harvesting strategies and incentives to encourage fishers to conduct surveys will be discussed.

**A NEW METHOD OF DEPLOYING COLLECTORS IN AREAS WITH FISHING ACTIVITIES.** Juan B. Peña, María J. Díez, and Carlos Saavedra. Instituto de Acuicultura Torre de la Sal (CSIC), E-12595 Ribera de Cabanes, Castellón, Spain.

We previously found a natural population of the Mediterranean scallop *Pecten jacobaeus* L. in an area between 50-m and 100-m depth in Castellón waters (Western Mediterranean). This is a fishing bed for some economically important fish species that are living on the sand and mud bottom around some rocky formations. However, there is a big rocky ground at a depth of 60–69 m where fishing boats do not fish. The main problem we have had in the last years is regarding the cutting of some of the collector's surface buoys by fishermen or curious people. The aim of this study is to improve and assure scallop collectors during the 7 months of settlement near the bottom. The surface water temperature in Castellón reaches 26°C to 27°C during summer, and usually collectors are removed in October to November, when the water temperature decreases to 18°C to 20°C. Until now the standard collector line has a surface buoy and a weight in the end of the rope to keep it tense. Bags are attached near the weight. In this study we compared the standard collector line with a new collector line formed by a rope of 90 m that had a weight on both ends and some small subsurface buoys in the central part of the rope. This new design kept a curved form with the upper arch at approximately 48 m from the sea surface. In this way, fishermen cannot see the buoys and the presence of collectors. A series of 14 collector bags sepa-

rated 70 cm between them and 3 m from the weight were attached to the rope. The strings of collectors were positioned April 30 and retrieved November 28, 2004. The collector lines were placed on the rocky bottom and their geographical position registered by a (global positioning system) GPS. In November the geographical position of each line was detected by the GPS and the boat sonar; then, collector lines were retrieved by an iron gadget with many spikes in order to catch the submerged curved rope. Seven species of pectinids were identified from the collectors. The most abundant was *Aequipecten opercularis* (55.5%), but other species were present and scarce. Most of them were without commercial value: *Crassadoma multistriata* (2.1%), *Mimachlamys varia* (0.1%), *Paltilium incomparabile* (32.7%), *Perapecten commutatus* (0.2%) and *Pseudamussium clavatum* (4.4%), with the exception of the king scallop *Pecten jacobaeus* (4.9%). Other species of bivalves were identified and counted, because of their number and size inside the bag: *Pteria hirundo* (18%) and *Atrina fragilis* (2.7%), but small-sized bivalves, were rejected without being counted. No significant differences were observed between the spat settlement on the standard collectors and the new collector, because both had 14 bags and were placed in the same area at the same time. The pectinid spat number per collector bag was on average 60.2 *Aequipecten opercularis* and 5.3 *Pecten jacobaeus* on the standard collectors and 60.8 and 5.5 on the arched collectors. The mean size of *Aequipecten opercularis* spat on standard collectors was  $17.34 \pm 0.19$  mm (range 10.5 to 27.2 mm) and on the arched collectors was  $16.87 \pm 0.11$  mm (range 8.5 to 29.9 mm). The mean shell height of *Pecten jacobaeus* spat was  $17.46 \pm 0.41$  mm (range 9.3–24.5 mm) and  $17.15 \pm 0.28$  mm (range 7.9–26.6 mm), respectively. The new method of deploying collectors gave a similar settlement of scallop spat than the standard collectors, but the novelty is in the placement without surface buoys and the retrieval by means of GPS and sonar. Therefore, the new arched line collector is a good method for catching pectinid spat in areas where the surface buoys from the collector strings can be cut or stolen by fishermen or curious sport fishermen, or even, by coast guards that think the ropes may contain drugs or other contraband goods.

**REPRODUCTION OF *NODIPECTEN SUBNODOSUS* (SOWERBY, 1835) IN SUSPENDED CULTURE IN THE GUERRERO NEGRO LAGOON, BAJA CALIFORNIA SUR MEXICO.** E. Pérez-DeLeón,<sup>1</sup> M. Arellano-Martínez<sup>2</sup> and B. P. Ceballos-Vázquez.<sup>2</sup> <sup>1</sup>Universidad Autónoma de Baja California Sur; <sup>2</sup>Centro Interdisciplinario de Ciencias Marinas, Instituto Politécnico Nacional, La Paz, B.C.S., México

Knowledge of the reproductive cycle of economically important marine invertebrates is basic to culture activities and management of natural stocks. Since 1997, an increasing interest in this species was observed especially because of its high potential for aquaculture. Documentation of the reproductive biology of *N. sub-*

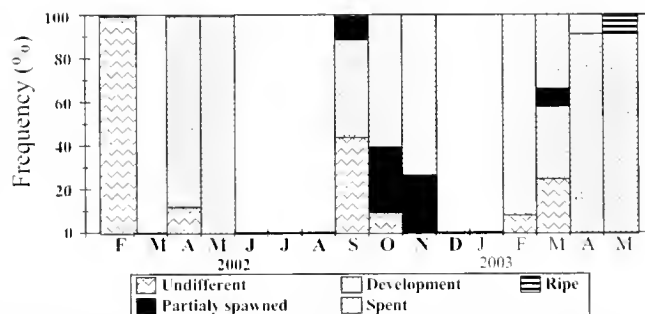


Figure 1. Reproductive cycle of *Nodipecten subnodosus* in suspended culture system in the Guerrero Negro lagoon, B.C.S. Mexico.

*nodosus* is scarce (Reinecke-Reyes 1996, Racotta et al. 2003, Arellano-Martínez et al. 2004). This study analyzes the reproduction time of *N. subnodosus* in suspended culture conditions in the Guerrero Negro lagoon at its northern geographical limit of distribution. Lion's paw scallop spat produced in the hatchery laboratory of Centro de Investigaciones Biológicas del Noroeste in La Paz B.C.S., Mexico were used in the study. In August 2001, 2-month-old scallops ( $\approx 3$  mm in shell height, HL) were seeded in the Guerrero Negro lagoon ( $27^{\circ}56'$  and  $28^{\circ}06'N$  and  $114^{\circ}02'$  and  $114^{\circ}09'W$ ). Nestier cages were suspended at 6-m depth near the bottom. Density was adjusted to maintain free the 50% of tray. HL of the scallops was registered at roughly monthly intervals over a period of 19 months. Since February 2002, between 5 to 12 scallops were sampled randomly each month. Gonads of these scallops were fixed in a 10% formalin solution for histological analysis. Additionally, water temperature and chlorophyll *a* concentration were recorded at each sampling time. Although there were no samples in all months, the tendency observed in the reproductive cycle (Fig. 1) agreed with previous reports for *N. subnodosus* (Reinecke-Reyes 1996, Racotta et al. 2003, Arellano-Martínez et al. 2004). It showed a clear seasonality related to the water temperature, ripening when temperature began to increase (April,  $18^{\circ}C$ ). Spawning occurs with high concentration of chlorophyll *a* and when temperature started to decrease (September) (Fig 2). The histological analysis shows that the first gonad development

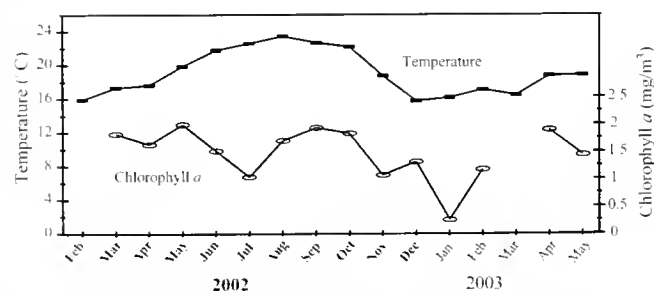


Figure 2. Monthly variation in water temperature and chlorophyll *a* concentration in the suspended culture system in the Guerrero Negro lagoon, B.C.S. Mexico.

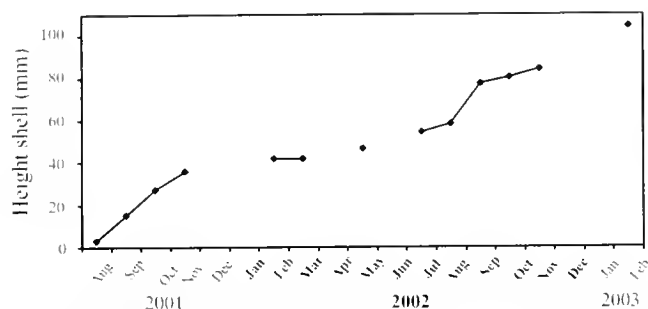


Figure 3. Average shell height reached at each sampling date in the suspended culture system in the Guerrero Negro lagoon, B.C.S. Mexico.

started when mean shell height was 50 mm (April, 9 months old). The earlier mentioned agreed with observations in other areas on the Pacific coast. In a wild population from Ojo de Liebre lagoon (adjacent to Guerrero Negro lagoon) scallops started the gonad development at 51-mmHL (8 months old, approximately) (Arellano-Martínez et al. 2004). In a culture system in Bahía Magdalena the gonad development started in scallops at 52-mmHL (9 months old) (Racotta et al. 2003). On the other hand, the gonad development coincided with a period of minimum increment in shell height (Fig 3), probably because the energy now is channeled to reproduction more than growth. According to the earlier mentioned and considering that *N. subnodosus* reaches large sizes (218-mmHL), it is evident that the lion's paw scallop is reproductively precocious with early gonad development before the first year of age.

### Acknowledgments

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**CHARACTERIZATION OF THE IMMUNE RESPONSE OF THE LION'S PAW SCALLOP *NODIPECTEN SUBNODOSUS*.** Erick R. Ramírez-Castillo,<sup>1</sup> Bertha P. Ceballos-Vázquez,<sup>1</sup> Antonio Luna-González<sup>2</sup> and Marcial Arellano-Martínez.<sup>1</sup> <sup>1</sup>Centro Interdisciplinario de Ciencias Marinas, Instituto Politécnico Nacional, La Paz, B.C.S. México; <sup>2</sup>Universidad Autónoma de Baja California Sur, La Paz, B.C.S. México.

The hydrolytic effects of the different lysosomal enzymes are one of the most important mechanisms in the degradation of pathogenic organisms inside or outside the hemocytes in bivalve mollusks. Therefore, the enzymatic activity in hemolymph has been extensively studied as indicator of the immune capacity in many species of bivalves. The objective of this work is to analyze the immune response of the lion's paw scallop *Nodipecten subnodosus* to the challenge with *Vibrio alginolyticus* strain APSA2, through the characterization of the enzymatic activity (lysosomal enzymes). Before the experiment it was necessary to determine the sublethal dose of *V. alginolyticus*. It was determined using 5 lots with 8 organisms each one and testing 5 concentrations from  $10^2$  to  $10^6$  UFC/mL during a period of 10 days. Another lot served as control, which was injected with saline solution. The sublethal dose was established at  $10^5$  UFC/mL. Then, 5 lots of 6 organisms were injected with the sublethal dose ( $10^5$  UFC/mL) and 4 organisms served as control by treatment using saline solution. Samples of 6 mL of hemolymph were obtained from the adductor muscle using a 5 mL sterile syringe after 6 and 24 h and 3, 6 and 10 d. In each case, hemocytes were quantified (hemocytes/mL) with a hemacytometer. Additionally, the diameter of the hemocytes was obtained using the SigmaScan Pro software (Version 5.0). To separate the hemocytes from the plasma the hemolymph was centrifuged at  $\times 500g/10$  min/ $4^\circ C$ . Once separated, they were resuspended and washed in buffer Tris HCl pH 6.5. Then hemocytes were broken by freeze-thaw to obtain the supernatants (HLS) of the cellular debris. Hydrolytic enzymes were determined in plasma and HLS with the API ZYM kit (BioMérieux) and lysoplate assay. Enzymatic activity was reported in relation with the protein con-

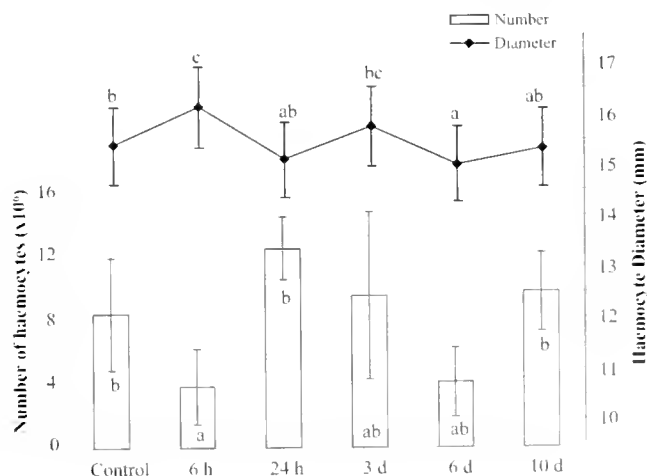


TABLE I.  
Enzymatic activity determined in HLS and plasma. Values shown are means ( $\pm$ SD) of 2 replicates (APIZYM kit, U hydrolysed substrate in nM mg<sup>-1</sup> protein) and 4 (lysoplate assay, U mg<sup>-1</sup> protein).

Enzymes	Control		6 Hours		24 Hours		3 Days		6 Days		10 Days	
	HLS	Plasma	HLS	Plasma	HLS	Plasma	HLS	Plasma	HLS	Plasma	HLS	Plasma
Phosphatases												
Acid phosphatase	1.97 $\pm$ 0.5	3.87 $\pm$ 0.8	2.89 $\pm$ 0	0 $\pm$ 0	3.11 $\pm$ 0	1.37 $\pm$ 0	2.09 $\pm$ 0	0 $\pm$ 0	1.84 $\pm$ 0	1.42 $\pm$ 0	2.7 $\pm$ 1.3	0 $\pm$ 0
naphthol	3.44 $\pm$ 2.3	1.34 $\pm$ 1.05	2.89 $\pm$ 0	1.37 $\pm$ 0	3.11 $\pm$ 0	0 $\pm$ 0	2.09 $\pm$ 0	0 $\pm$ 0	3.69 $\pm$ 0	0 $\pm$ 0	1.85 $\pm$ 0	2.37 $\pm$ 0
Phosphohydrolase												
Alkaline												
phosphatase	2.06 $\pm$ 0.6	4.76 $\pm$ 0.0	1.44 $\pm$ 0	0 $\pm$ 0	1.55 $\pm$ 0	0 $\pm$ 0	1.56 $\pm$ 0.7	22.62 $\pm$ 0	0.92 $\pm$ 0	0 $\pm$ 0	1.85 $\pm$ 0	0 $\pm$ 0
Esterases	3.34 $\pm$ 2.5	11.62 $\pm$ 5.4	2.89 $\pm$ 0	10.98 $\pm$ 0	12.47 $\pm$ 0	6.86 $\pm$ 5.8	4.18 $\pm$ 0	5.65 $\pm$ 0	1.84 $\pm$ 0	14.22 $\pm$ 4	5.55 $\pm$ 2.6	9.48 $\pm$ 0
Lipases	0 $\pm$ 0	2.38 $\pm$ 0.4	2.89 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	1.37 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	9.48 $\pm$ 0
Proteases	15.36 $\pm$ 0.5	8.19 $\pm$ 1	23.16 $\pm$ 0	6.86 $\pm$ 5.8	24.95 $\pm$ 0	16.48 $\pm$ 0	16.74 $\pm$ 0	5.65 $\pm$ 0	14.76 $\pm$ 0	4.26 $\pm$ 2	14.82 $\pm$ 0	7.11 $\pm$ 3.3
Leucyl arylamidase												
Valil arylamidase	1.47 $\pm$ 0.4	0 $\pm$ 0	1.44 $\pm$ 0	0 $\pm$ 0	1.55 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	1.38 $\pm$ 0.6	0 $\pm$ 0	1.38 $\pm$ 0.6	0 $\pm$ 0
Cistil arylamidase	2.46 $\pm$ 0.9	0 $\pm$ 0	2.17 $\pm$ 1	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	1.04 $\pm$ 0	0 $\pm$ 0	1.38 $\pm$ 0.6	0 $\pm$ 0	1.38 $\pm$ 0.6	0 $\pm$ 0
$\alpha$ Chymotripsina	0.59 $\pm$ 0.2	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0.92 $\pm$ 0	0 $\pm$ 0
Glycosidases												
$\beta$ Galactosidase	1.77 $\pm$ 0.5	0 $\pm$ 0	1.44 $\pm$ 0	0 $\pm$ 0	3.11 $\pm$ 0	0 $\pm$ 0	1.04 $\pm$ 0	0 $\pm$ 0	1.38 $\pm$ 0.6	0 $\pm$ 0	0.92 $\pm$ 0	0 $\pm$ 0
$\beta$ Glucoronidase	0.49 $\pm$ 0.1	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	1.55 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
Lysozyme	3134.72	3774.7	1348.806	5302.063	3028.451	4510.357	1717.554	5173.251	2481.8	6129.855	2796.738	4912.974

centration of samples. The concentration of protein was determined by the Bradford method. The samples of each time were pooled taking 400  $\mu$ L/sample. The ANOVA was used to assess significant differences between times. To compare the quantity of hemocytes, the mean of all controls was obtained. The quantity of hemocytes was significantly lower (ANOVA,  $P < 0.05$ ) 6 h after challenge, whereas the hemocytes were significantly larger (ANOVA,  $P < 0.05$ ) (Fig. 1). The quantity and diameter of hemocytes in treatments were negative and significantly correlated ( $r = -0.82$ ,  $P < 0.001$ ). Of the 19 enzymes detected by the API ZYM kit, only 11 can be characterized in SLH and 5 in plasma (Table 1). The enzymatic activity was significantly higher (ANOVA,  $P < 0.05$ ) in hemocytes than in plasma. The enzymes with higher activity were esterase and leucyl arylamidase in both, SLH and plasma. Lysozyme-like activity did not show significant differences (ANOVA,  $P < 0.05$ ) between the sampling times. However, in plasma the lysozyme-like activity was significantly higher than in HLS. The infection was controlled in almost all bacterial concentrations. However, the concentration of  $10^6$  UFC/mL exceeded the capacity of immune response producing a total mortality of scallops 72 h after challenge. With the sublethal dose ( $10^5$  UFC/mL) at 6 h, 3 d, and 6 d, animals showed lower hemocyte numbers. This may be due to their lysis and/or movement into tissues (diapedesis) provoked by bacteria while fighting infection. However, at 24 h hemocyte number was higher than the control and cell diameter was lower. It seems that hematopoiesis was induced by the bacterial infection because it could be seen by the number and hemocyte diameter. Hematopoiesis had been wound-induced in the pearl oyster *Pinctada fucata martensii*, where proliferation occurs in the connective tissues of the stomach and intestine. On the other hand, the enzymatic activity determined by the API ZYM kit did not show a clear tendency. Enzymes with higher activity, esterase and leucyl arylamidase, are the responsibility of the change in the conformation of the cell wall of the bacteria. Also, lysozyme acts destroying the bacterial cell wall, making the bacterial degradation easier. Lysozyme-like activity was higher in plasma than in hemocytes, especially in the treatments with bacteria. The higher enzyme concentration in plasma suggests that lysozyme was released from hemocytes into plasma during the pathological stress caused by *V. alginolyticus*.

**COMPARATIVE GROWTH OF THREE SCALLOP SPECIES IN MEXICO—(ARGOPECTEN VENTRICOSUS, NODIPECTEN SUBNODOSUS AND EUVOLA = PECTEN VOGDESI).** César A. Ruiz-Verdugo<sup>1</sup> and Mignel Robles-Mungaray.<sup>2</sup> <sup>1</sup>Laboratorio Experimental de Acuicultura, Departamento de Ingeniería en Pesquerías, Universidad Autónoma de Baja California Sur, Ap. Postal 19-B, La Paz, Baja California Sur, México, 23000; <sup>2</sup>Sea Farmers S.A de C.V., Av Obregon # 525 pte. col centro, Los Mochis, Sinaloa, México.

In the past years, efforts have been made in Mexico to diversify aquaculture of marine bivalves, especially scallops. Several studies in the past 15 years have focused on the culture of the Pacific

calico scallop (*Argopecten ventricosus*, Sowerby II, 1842), however, only a single company in the state has been successful in producing commercial quantities over a long time period. One of the principal limitations for the culture of the Pacific calico scallop is its small size (up to 60–80 mm), this implies that large amounts of organisms must be grown, and that the commercial value of the meats is low in comparison to species with larger adductor muscles. In addition to the Pacific calico scallop, there are two species of pectinid of commercial importance in Mexico, the lion's paw scallop (*Nodipecten subnodosus*) and the flying scallop (*Euvola* = *Pecten vogdesi*), which are larger (*N. subnodosus* up to 250 mm and *P. vogdesi* 130 mm) and therefore reach higher market values. *N. subnodosus* is one of the largest pectinids worldwide, and some basic growth studies and feasibility of different culture methods have been done in the past years. For *E. vogdesi* only little information exists. No comparative analysis of growth of all three species under the same culture conditions has as yet been undertaken. Therefore, this study attempts to compare growth of the three native pectinids under cold conditions in Magdalena Bay and warm water conditions in La Paz Bay. The spat of *Argopecten ventricosus* and *Nodipecten subnodosus* was produced in the Laboratory of Larviculture CIBNOR (Biological Research Center of the Northwest, S.C. La Paz, B.C.S.). The juveniles of Pacific calico and Lion's paw scallop averaged 17.0 mm and 19.0 mm shell height respectively. The spat of *Euvola vogdesi* was produced in the Laboratory of Experimental Aquaculture at the University of Baja California Sur (UABCS) and averaged 16.0 mm shell height at the beginning of the experiment. The spat was cultured in 2 sites, Rancho Bueno (Magdalena Bay) and Pichilingue (La Paz Bay) (Fig. 1). Oyster trays suspended from a long line were used to culture the organisms, 40 organisms of each one of the three species of pectinid were placed in the same tray, using the replicates total. Growth and survival was measured monthly from February to June of 2002. On each occasion, shell height of 30 organisms of each species was height measured using a digital vernier ( $\pm 0.01$  cm). Results of this experiment are shown in Figure 2. *N. subnodosus* shows higher growth at both culture sites. The best growth for all three species was reached in Magdalena Bay,

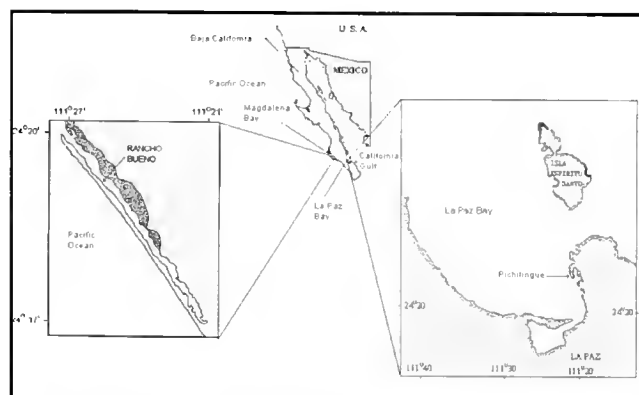


Figure 1. Cultivation area on Baja California Peninsula.

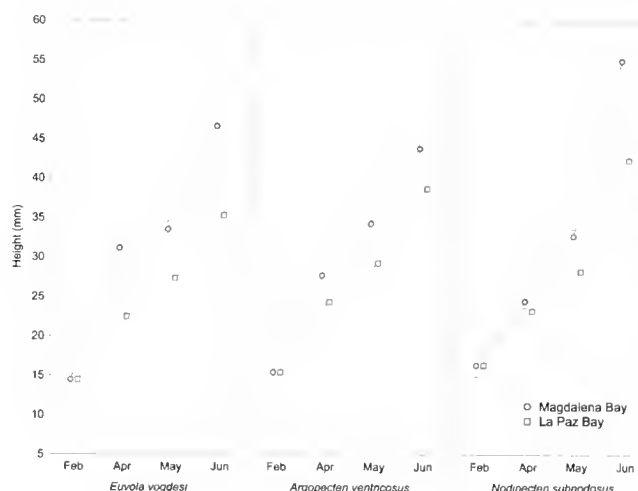


Figure 2. Growth of *Euvola vogdesi*, *Argopecten ventricosus* and *Nodipecten subnodosus* cultures in Magdalena Bay and La Paz Bay. Means at month are indicated with their standard errors.

where lower temperatures and a higher primary productivity are found than in La Paz Bay. Final average size was  $55.02 \pm 0.45$  mm for *N. subnodosus*,  $46.61 \pm 0.58$  mm for *E. vogdesi*,  $43.8 \pm 0.45$  mm for *A. ventricosus*. The breeders of *N. subnodosus* and *A. ventricosus* came from the Pacific side of the Peninsula while breeders of *E. vogdesi* were from the Gulf of California. The results show that *N. subnodosus* and *E. vogdesi* are the most promising species for aquaculture development in the region. It is necessary to study the culture of *E. vogdesi* in the Pacific and of *N. subnodosus* in the Gulf of California, and to evaluate whether lion's paw spat resulting from breeders of the Gulf of California, shows better growth and survival under warm water conditions than organisms originating from the Pacific population.

**INFLUENCE OF DEPTH ON GROWTH AND SURVIVAL OF THE LION'S PAW SCALLOP *NODIPECTEN NODOSUS* (LINNAEUS, 1758) IN SOUTHERN BRAZIL.** Guilherme S. Rupp,<sup>1</sup> Gilberto C. Manzoni,<sup>2</sup> Micheline M. de Bem,<sup>3</sup> Lin H. L. Iwersen,<sup>3</sup> Celso C. Buglione Neto<sup>3</sup> and Erich Cerchiari.<sup>2</sup>  
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The Atlantic lion's paw scallop, *Nodipecten nodosus*, is the largest pectinid occurring in Brazilian waters and is considered a prime candidate species for aquaculture due to its size and commercial value. Whereas there are studies on the effects of environmental factors affecting growth and survival during nursery culture (Rupp et al. 2004a, 2005), little information is available about the growout phase in Brazil. An understanding of the effects

TABLE 1.

Mean shell height, adductor muscle wet weight, total somatic tissue wet weight, dry weight of fouling and percent survival (January 2005).

Depth	Shell Height (mm) (95% CI)	Adductor Muscle Wet Weight (g) (95% CI)	Total Somatic Tissue Wet Weight (g) (95% CI)	Dry Weight of Fouling (95% CI)	% Survival
3 m	70.19 (3.8)	9.80 (1.2)	21.93 (2.6)	17.3 (2.2)	82.2
9 m	63.79 (2.3)	6.04 (1.1)	13.97 (2.3)	10.7 (1.4)	84.1

of environmental factors associated with depth is essential to establish optimized culture strategies and therefore the basis for commercial aquaculture. This study was carried out to compare the effects of a wide array of environmental factors associated with depth on growth and survival of *N. nodosus* cultured in Santa Catarina State, Brazil. *Nodipecten nodosus* (Linnaeus, 1758) juveniles were hatchery-produced and transferred to sea-based nursery using techniques described by Rupp et al. (2004b, 2005). When a mean shell height of 18 mm was attained (February 2004), scallops were suspended on a sub-surface long-line at 3 and 9 m in triplicate lantern-nets at each depth. The experiment was carried out at a coastal aquaculture site (Armação de Itapocoroy) (26°46'S to 48°37'W), in Santa Catarina State, Brazil. Lantern nets were replaced on a monthly basis when live scallops were also counted and measured for shell height. Every other month a sample of 12–15 scallops/net were transferred to the laboratory for measurement of somatic and reproductive growth, as well as quantification of fouling organisms settled on the shells. Temperature was recorded hourly and Chlorophyll *a*, total seston, particulate organic matter, particulate inorganic matter and dissolved oxygen were measured twice a month. In May 2004, because of a large variation

TABLE 2.

Mean temperature, salinity, concentration of chlorophyll (*a*), PIM, POM, % PIM and turbidity.

Depth	Temp (°C)	Salinity ‰	Chlorophyll ( <i>a</i> ) (µg/L)	PIM mg/L	POM mg/L	% PIM	Turbidity NTU
3 m	22.6	33.78	2.77	3.35	0.95	75.8	2.73
9 m	22.3	34.31	2.8	6.83	1.16	84.6	5.13

in size and to mimic commercial operations, scallops were size-graded and those smaller than 22 mm were separated in different nets. The larger group was maintained at their respective depths in triplicate nets. Results are presented for the larger group of scallops grown from May 2004 to January 2005. Mean shell heights of the larger group in May 2004 were 40.61 mm and 37.28 mm at 3 and 9 m respectively, which were statistically similar (Anova,  $P < 0.01$ ). The mean number of scallops per lantern net was 243 at 3 m and 234 at 9 m, which represented 58% and 57% respectively of the total number of scallops before size selection. Table 1 shows the mean shell height, adductor muscle wet weight, total somatic tissue wet weight, and dry weight of fouling settled on shells, recorded after 8 months of suspended culture (January 2005). Percent survival was determined in relation to the number of scallops after size selection (May 2004). Table 2 shows the mean temperature, salinity, concentration of chlorophyll *a*, PIM, POM, % PIM in relation to total seston and turbidity. Figures 1 and 2 show the growth curves in shell height and adductor muscle wet weight, respectively. Growth was least at 9 m, where higher turbidity and higher PIM were recorded. A commercial size adductor muscle (mean wet weight of 6 g) was attained in September 2004 at 3 m, whereas at 9 m, it was only attained 4 months later.

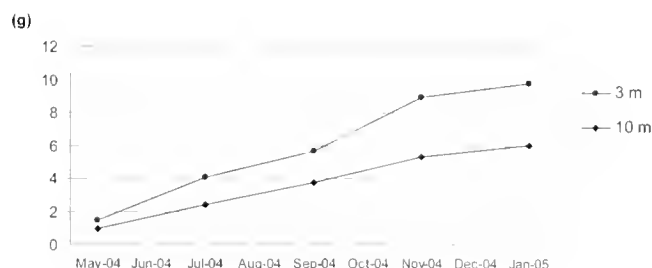


Figure 1. Shell height.

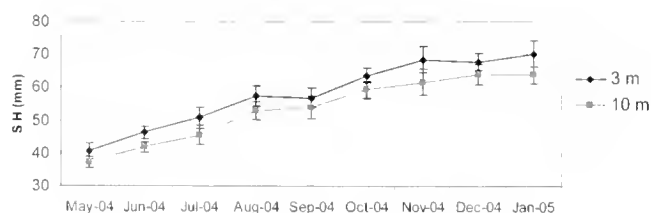


Figure 2. Adductor muscle wet weight.

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**MOLECULAR PHYLOGENETICS OF AMERICAN PECTINIDS.** Carlos Saavedra and Juan B. Peña. Instituto de Acuicultura de Torre la Sal-CSIC, Ribera de Cabanes, E-12595 Castellón, Spain.

Pectinids are one of the most conspicuous families of Bivalves living in the sea. In spite of its abundance and economic importance, the taxonomy and systematics of pectinids is not well known. Several different taxonomic classifications below the family level have been proposed in the past, most of them based on shell features of adult individuals. In a collection of papers, Waller argued that these were not good characters for taxonomy, and based his classification in features of the larval shell. He proposed that the family could be divided into 2 subfamilies, pectininae and chlamydiae. The problem of the classification of pectinids has been recently approached with the tools of molecular phylogenetics. Matsumoto and Hayashi (2000) studied a fragment of the mitochondrial cytochrome oxidase gene in 17 species. His analysis was based on amino acid sequences, and supported the existence of the two subfamilies proposed by Waller. Barucca et al. (2004) published a study of 23 species (16 genera), based on ca. 1000 nucleotides of the mitochondrial large and small ribosomal RNA subunits, supporting that subdivision as well. Most of the species studied by Matsumoto and Hayami were from the west Pacific, whereas most of the species studied by Barucca et al. were from other regions, mainly Europe. American species were poorly represented (3 species in Barucca et al.'s study, of which only one was exclusive of the American continent; 1 non-exclusive species in Matsumoto and Hayami's study). However, the American continent has a very specific pectinid fauna, with several exclusive genera. Many of these species have commercial interest as well. The phylogenetic relationships of these genera and species and its evolutionary history has been studied from a morphological and paleontological perspective, but rarely from a molecular phylogenetics perspective. A molecular phylogenetic study of these genera and of its relationships with pectinids from the other continents is highly desirable. Here we present a phylogenetic analysis of 10 American species belonging to 6 different genera: *Argopecten gibbus*, *A. purpuratus*, *A. ventricosus*, *Euvola ziczac*, *E. vogdesi*, *Hinnites giganteus*, *Nodipecten nodosus*, *N. subnodosus*, *Placopecten magellanicus* and *Zygochlamys patagonica*. We scored the same 16 S and 12 S ribosomal gene regions studied by Barucca et al., which allowed us to conduct a phylogenetic study of these taxa in the context of the world pectinid fauna.

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- BENTHIC RICHNESS IN SCALLOP BEDS—*ZYGOCHLAMYS PATAGONICA* (KING AND BRODERIP, 1832) AS PRIMARY SETTLEMENT SUBSTRATE.** Laura Schejter and Claudia Bremec. INIDEP, Instituto Nacional de Investigación y Desarrollo Pesquero, Paseo Victoria Ocampo 1, 7600, Mar del Plata, Argentina.
- The availability of a suitable substratum is one of the critical factors for the colonization of sessile species. Mollusc shells, decapod carapaces and sea urchin spines are several frequent surfaces used as hard substrata available for settlement of sessile organisms in soft bottoms. Epibiosis is the scientific name for this aquatic phenomenon of a non-symbiotic association between epibionts (organisms growing attached to a living surface) and basibionts (substrate organisms that are hosts to the epibionts) (Wahl 1989). Previous works on *Zygochlamys patagonica* (Diehl 1977, Walossek 1991, Rosso & Sanfilippo 1991, Sanfilippo 1994, Bremec & Lasta 2002, Bremec et al. 2003) mentioned a great variety of organisms, including calcareous algae, poriferans, hydrozoans, anthozoans, polychaetes, molluscs, cirripeds, foraminiferans, ascidians and bryozoans. This study summarizes available and new information of epibionts on *Zygochlamys patagonica* and assesses the contribution of this basibiont to increasing the species richness in scallop beds in the Argentine Sea. The checklist of epibionts is given. We compiled information from the bibliographic sources earlier cited that refer to epibionts on Patagonian scallops. Additionally, we give new results from material collected during monitoring cruises (INIDEP) carried out during 2001 (Hydrozoa, G. Genzano, pers. comm.) and 2003 (Porifera, Schejter et al. in prep., Polychaeta & Ascidacea, pers. observ.). The study area comprises between 39° S and 47° S in the Argentine Sea, along the 100-m isobath and in the north-patagonian coast (90–110 m) and the Magellan Strait (80m, 53°S). A total of 35 epibiont taxa was registered on *Zygochlamys patagonica* valves (Table 1). The presence of the sponge *Hymedesmia* (*Stylopus*) *longurius* (Hentschel, 1914) is a new record for the Argentine Sea, the species was only known for Antarctic waters (Schejter et al., in prep.). The hydroids *Lafaea fruticosa* (M. Sars, 1851) and *Phiallela chilensis* Hartlub, 1905 were found as secondary level epibionts (epibionts of other *Z. patagonica* epibionts, mainly other hydroids). Other epizotic organisms were small isopods and amphipods, found as free living organisms between the crevices developed by the association of epibionts. The mollusc *Calyptrea pileolus* (d'Orbigny, 1841) and the sea cucumber *Psolus patagonicus* (Eckman, 1925) were closely associated to the scallop valves, and unidentified small egg capsules of gastropods were also occasionally found (personal observation).
- A total of 82 species were identified in the Patagonian scallop assemblage in the Argentine Sea, including the deeper fishing grounds along the 100 m isobath and the coastal north-patagonian beds Sea Bay and Tres Puntas, 16 of which were indicated as scallop epibionts (Bremec & Lasta 2002). This region is characterized by soft bottoms and the scallops constitute the available

TABLE 1.  
Epibionts on *Zygochlamys patagonica*.

N	TAXA	Reference
1	Foraminifera	1, pers. obs.
2	<i>Iophon</i> sp.	2, 3, 4
3	<i>Hymedesmia</i> ( <i>Stylopus</i> ) <i>longurius</i> (Hentschel, 1914)	4
4	<i>Clathria</i> sp.	4
5	<i>Grammaria magellanica</i> Allman, 1888	5
6	<i>Synplectoscyphys subdichotomus</i> (Kirchenpaver, 1884)	5
7	<i>Plumularia setacea</i> (Linné, 1758)	5
8	<i>Lafoea fruticosa</i> (M. Sars, 1851)	5*
9	<i>Phiallela chilensis</i> Hartlaub, 1905	5*
10	<i>Acyonium</i> sp.	2, 3
11	Actiniaria	3
12	<i>Hiatella solida</i> (Sowerby, 1834)	2, 3
13	<i>Zygochlamys patagonica</i> (King & Broderip, 1832)	3
14	<i>Serpula narconensis</i> Baird, 1865	1, 2, 3, 6
15	<i>Idanthyrsus armatus</i> Kinberg, 1867	1, 2, 3, 6
16	<i>Protolaeospira</i> ( <i>P.</i> ) <i>lebruni</i> Harris, 1969	1
17	<i>Romanchella perrieri</i> Caullery & Mesnil, 1879	1
18	<i>Romanchella</i> cf. <i>inventis</i> (Harris, 1969)	1
19	Other Serpulidae	1
20	Other Spirobidae	1
21	Sabellidae	2, 3
22	<i>Chaetopterus variopedatus</i> (Ranier, 1807)	pers. obs.
23	<i>Ornatoscalpellum</i> sp.	2, 3
24	"barnacles"	1, 7
25	<i>Magellania venosa</i> (Solander, 1786)	2
26	<i>Terebratela dorsata</i> (Gmelin, 1790)	2
27	<i>Alloccarpha incrustans</i> (Herdman, 1886)	3, 6, 8
28	<i>Cnemidocarpa nordenskjöldi</i> (Michaelsen, 1898)	3, 6, 8
29	<i>Paramolgula gregaria</i> (Lesson, 1830)	pers. obs.
30	<i>Didemnum</i> sp.	2
31	<i>Acyonidium australe</i> Waters, 1904	7
32	Membraniporidae	2
33	<i>Porella</i> sp.	2
34	Other Bryozoa	2
35	Green algae	1, 7

hard substrate for the settlement of sessile invertebrates; at least 33 taxa are mostly epibionts on scallops according to our results. Green algae and barnacles only encrusted scallops from the Magellan Strait (Rosso & Sanfilippo 1991, Sanfilippo 1994). It is remarkable that recruits of Patagonian scallops only settled on living scallops (Bremec et al. 2003, Bremec & Schejter this Workshop), as well as the other epibionts listed in Table 1. Free shells provided substrate to egg capsules of *Fusitriton magellanicus* Rodding, 1798, *Odontocymbiola magellanica* Gmelin, 1791 and *Adelomelon ancilla* (Solander, 1786) (Bremec et al. 2003) and occasionally sponges. Among other frequent and conspicuous organisms of the benthic assemblage, the spider crab *Libinia* *granaria* (Schejter & Spivak, in press), the gastropods *Fusitriton magellanicus*, *Odontocymbiola magellanica* and *Adelomelon ancilla* presented encrustations, not all taxa shared with scallops

(pers. obs.). It is known that most of the epibiont species in the world are not species-specific (Wahl & Mark 1999) even though epibionts are almost restricted to scallop valves in the Patagonian scallop beds. The contribution of *Zygochlamys patagonica* to increasing the benthic species richness is greatly favored by its dominance, both in abundance and biomass.

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**BENTHIC COMMUNITY ASSEMBLAGE ASSOCIATED WITH THE PATAGONIAN SCALLOP FISHERY IN RE-CLUTAS BED, 39°S, ARGENTINA, BETWEEN 1998 AND 2003—COMPARISON OF DISTURBED AND UNDISTURBED AREAS.** Laura Schejter, Claudia S. Bremec and Daniel Hernández. INIDEP, Instituto Nacional de Investigación y Desarrollo Pesquero, Paseo Victoria Ocampo 1., 7600, Mar del Plata, Argentina.

Fishing provokes the removal of a huge biomass of target and non-target species. Trawling or dredging over the marine bottom produces an important effect on the ecosystem, damaging not only

the substrate but also the invertebrates associated with the bottom. These modifications could cause destruction of the benthic habitat and long and short-term changes in the composition and structure of the associated fauna (National Research Council, 2002). The Patagonian scallop, *Zygochlamys patagonica* (King & Broderip, 1832), is distributed in the Magellanic Biogeographic Province, and it is the target species of a recent fishery in Argentina along the 100 m isobath (Lasta & Bremec 1998). The Reclutas bed is located between 39°00'–39°30'S and 55°45'–56°05'W. An exclusion area (39°20'–39°30'S and 55°52'–56°00'W, not allowed to commercial fishing) was established inside this bed in 1996. The aim of this study is to compare the composition and structure of the assemblage in the scallop bed Reclutas, in areas subjected to fishing effort and in the exclusion area, between 1998 and 2003, in order to identify short-term faunistic changes. A total of 83 samples were collected at Reclutas bed during assessment research cruises (R/V Capitán Cánepa, INIDEP) in 1998, 2001, 2002 and 2003, with a non-selective dredge of 2.5 m wide (efficiency 43%; Valero, 2002) following a sampling design of stations spaced regularly at 5 nm intervals. Additionally, 8 samples in 1998, 6 in 2001, 9 in 2002 and 3 in 2003 were also taken in the exclusion area of the same bed. Identification of organisms, abundance and biomass data were obtained on board (1998 and 2001) and at the laboratory (2002 and 2003). Data indicate abundances and biomasses of organisms per 100 m<sup>2</sup>. Multivariate analysis (ANOVA, PRIMER software) was applied. Hierarchical and multidimensional analysis (Bray-Curtis similarity index, fourth root transformation) and SIMPER test were developed to establish similarity between stations and species assemblages. ANOVA was used to test differences between areas (fished-REX) and years (1998–2003); transformation "log(x + 1)" was used for biomass values. ABC curves were used to detect disturbance levels in all years and areas. Richness in the fishing area (F) ranged from 37 to 56 and number of taxa per station varied between 5 and 29, both varying in different years. In the exclusion area (REX), richness ranged from 23 to 40 and number of taxa per station varied between 8 and 25. Differences in richness are mainly due to differences in sampling effort (that was higher in the last 2 years) and to the expertise acquired in identification of taxa recorded. In general terms, echinoderms were always dominant in biomass both in F and REX. Four invertebrate taxa (*Austrocidaris canaliculata*, *Cosmasterias lurida*, *Ctenodiscus australis* and Porifera) were closely associated with the Patagonian scallop in every year and area studied, representing approximately 45% (or more) in biomass contribution (SIMPER analysis) to the community assemblage. The addition of other 7 common taxa (*Ophiactis asperula*, *Ophiacantha vivipara*, *Libidoclaca granaria*, *Actinostola crassicornis*, *Fusitriton magellanicus*, *Calyptiraster* sp. and *Flabellum* sp.) increases the biomass contribution to approximately 70% (up to 80% in 1998 in F). ABC curves showed that F presented a moderately disturbed pattern in the 4 years, while REX, in 1998 showed a moderately disturbed area, but in 2002 biomass

curve is above abundance curve, in accordance with an undisturbed area. Bremec et al. (2000) found that at least eight samples are needed to get representation of most of the species in the Patagonian scallop benthic community. For that reason, samples from 2001 and 2003 in REX are not considered for the ABC analysis. Total biomass and scallop biomass in F differ significantly from REX considering all years (ANOVA: F = 5.491,  $P = 0.021$  and F = 9.72,  $P = 0.0023$ , respectively). In both cases, biomasses were higher in REX. Faunal biomasses (excluding scallops) did not differ significantly between years or areas. In this study we found that the Patagonian scallop assemblage at Reclutas bed was persistent after 9 years of fishing activity, between 1995 and 2003 (see Bremec & Lasta 2002 for 1995 data). Changes in species richness detected since 1995, baseline condition, are attributed to the sampling procedure. Regarding comparisons between fished and non-fished areas, the total number of species was lower in the latter; but this could be attributed to the number of samples analyzed, what permits the occurrence of the majority of the species and also to the size of REX, which could make sampling the rare or occasional species difficult. ABC curves in REX, however, seem to show a recuperation of biomass in time. Short-term observations show differences in scallop biomass only between areas (F and REX); the fishing strategy carried out during the study period in Reclutas bed did not produce significant differences in the biomass of by-catch taxa.

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**SCALLOPS AND ALGAL TOXINS REVISITED. Sandra E. Shumway.** Department of Marine Sciences, UCONN, 1080 Shennecossett Road, Groton, CT 06340, USA.

The interactions between scallops and toxic algae are complex and often unique to the algal species, scallop species and geographic region under consideration. In geographic regions where

only the adductor muscle has been traditionally marketed, algal toxins have not posed a significant threat to human health. In regions where both whole and "roe-on" scallops are consumed there is a need for careful monitoring of product. Recent increases in aquaculture and the marketing of whole scallops have rekindled the discussions on sequestering and biotransformation of algal toxins. There is a general increased awareness of the potential dangers of marketing and consuming whole scallops and consequently, more monitoring programs now include scallops as part of their regular sampling regimes. As scallop culture and the desire to market scallop parts other than the adductor muscle continue to expand globally, so will the need to establish comprehensive and affordable monitoring programs for algal toxins.

**APPLICATION OF MULTIBEAM BATHYMETRY AND SURFICIAL GEOLOGY TO THE SPATIAL MANAGEMENT OF SCALLOPS (*PLACOPECTEN MAGELLANICUS*) IN SOUTHWEST NOVA SCOTIA.** Stephen J. Smith,<sup>1</sup> Gerard Costello,<sup>2</sup> Vladimir E. Kostylev,<sup>3</sup> Mark J. Lundy<sup>1</sup> and Brian J. Todd.<sup>3</sup> <sup>1</sup>Department of Fisheries and Oceans, Invertebrate Fisheries Division, Bedford Institute of Oceanography, Dartmouth, Nova Scotia B2Y 4A2, Canada; <sup>2</sup>Department of Fisheries and Oceans, Canadian Hydrographic Service, Bedford Institute of Oceanography, Dartmouth, Nova Scotia B2Y 4A2, Canada; <sup>3</sup>Geological Survey of Canada (Atlantic), Natural Resources Canada, Bedford Institute of Oceanography, Dartmouth, Nova Scotia B2Y 4A2, Canada.

The management of scallop fisheries of the Scotian Shelf, Bay of Fundy and the Canadian side of Georges Bank currently include spatial components. Areas have been identified for seasonal closures to avoid gear conflicts, different size/meat weight or harvesting restrictions due to spatial variation in growth rates and closures to protect recently settled abundant year-classes. However, the development of reference points for harvest levels have generally taken a dynamic pool approach to their definition ignoring spatial patterns in abundance, age distribution, growth rates and settlement patterns. Recent work has identified large differences in growth rates within relatively short distances for scallop beds in the Bay of Fundy. Ignoring spatial heterogeneity of scallop populations ignores the fact that fishing also concentrates on spatial patterns reflecting growth rates and densities. Canada is committed to developing fine-scale maps of the bathymetry and surficial geology of its continental shelf areas to provide the basis for the management and conservation of the biological and mineral resources within its national jurisdiction. Bottom habitat maps developed from multibeam bathymetry, backscatter strength and geological sampling have been used by the offshore scallop industry to allocate fishing effort. The main advantages from using these maps

to the fishing industry has been to reduce fishing effort in areas that are marginal for scallops or difficult to tow gear in, increase overall catch rates by concentrating effort in areas identifiable as scallop habitat and reduce costs associated with fishing. However, these maps have not been used to develop fine-scale management plans that are attuned to the spatial heterogeneity of the scallop populations. Scallop grounds off of southwest Nova Scotia have recently been mapped using multibeam sonar technology in a joint project partnership of the Department of Fisheries and Oceans, Natural Resources Canada and the local scallop fishing industry. One of the major objectives of this project is to collect spatial information on scallop distribution, growth and size composition, bycatch data, as well as fine-scale bathymetry and surficial geology for the purposes of developing spatially based fisheries management plans. There are two phases for the work on this objective. In the first phase, multibeam backscatter strength data, geological and benthic sampling data will be used to develop habitat maps of the area. At the same time, spatial patterns of scallop growth, densities and size composition obtained from concurrent scallop drag surveys will be related to these habitat attributes. All of this information does not in itself determine what the objectives of spatial management are and the potential advantages of spatial management over the standard approach will be explored in the second phase of the project.

**TOWCAM—TOWED CAMERA ARRAY FOR VIDEO/ STILL BENTHIC SURVEYS.** Stephen J. Smith,<sup>1</sup> D. McKeown,<sup>1</sup> M. Lundy,<sup>1</sup> D. Gordon,<sup>1</sup> J. Anderson,<sup>2</sup> M. Strong<sup>3</sup> and M. Power.<sup>3</sup> <sup>1</sup>Dept. Fisheries and Oceans, Bedford Institute of Oceanography, Dartmouth, Nova Scotia B2Y 4A2, Canada; <sup>2</sup>Dept. Fisheries and Oceans, Northwest Atlantic Fisheries Centre, St. John's Newfoundland A1C 5X1, Canada; <sup>3</sup>Dept. Fisheries and Oceans, Biological Station, St. Andrews, New Brunswick E5B 2L9, Canada.

Towcam is a bottom-following optical survey system developed by the department of fisheries and oceans at the Bedford Institute of Oceanography in Dartmouth, Nova Scotia. This system is being used for conducting video and photographic surveys of benthic communities and has been routinely deployed from vessels as small as 19.8 m (LOA). This poster shows examples of recent applications of this new survey tool for a number of benthic studies. Field work on scallop beds was conducted in 2002 and 2003 in the Bay of Fundy to assess the feasibility of operating TowCam from a small vessel (CCG J. L. Hart). Whereas there was a great deal of suspended material in the water, the quality of the digital stills was impressive. This technology will allow us to estimate *in-situ* densities of scallops along with size compositions unaltered by the selectivity effects of the scallop drags that we currently use for our surveys. In addition, estimates of size and numbers of

associated species and qualitative information on bottom type can also be obtained from these pictures—data not usually measured when scallop drags are used to survey the area. The Department of Fisheries and Oceans (DFO) and the Geological Survey of Canada, Atlantic (GSCA) are conducting a project to study the spatial utilization of benthic habitat by demersal fish on the Scotian Shelf. Field studies are being conducted at six sites selected using historical groundfish survey data to identify areas with the highest and lowest probability of encountering juvenile haddock. Towcam is a critical tool in the field program and is used off the C.C.G.S. Hudson to collect video and digital still imagery of the seabed along 5-km transects. This video and still imagery along with other data are being analyzed to look at the interrelationships between surface-dwelling benthic invertebrates, fish and seabed habitat characteristics over spatial scales ranging from kilometers down to a few centimeters. Herring eggs adhere to the bottom substrate after spawning takes place. Because of the timing of this survey (August) no herring spawning had yet taken place on German Bank (although there is now substantial reported spawning activity from the fishing fleet over the last few days) and so no herring spawn was observed. We believe that this technology could be very useful in determining the substrate on which spawning occurs and in mapping areas of successful spawning activity. The images captured are sufficient to be able to identify this with ease, but there would be the issue of bottom suitability to safe TowCam operations because much of the spawning area is of highly irregular bottom. The Gulf of Maine Crustacean Fisheries Research Section at St Andrews N.B. has tested Towcam for capturing benthic geo-referenced video footage used to determine lobster distribution and abundance in the shoal waters of Lobster Bay Nova Scotia. A large area could be surveyed in a single day because of the fast towing speed and the large camera footprint rendered when the altitude was between 2 and 3 meters above the bottom. For quantitative purposes, the option of being able to accurately calculate the footprint size at a given time from laser indexing, pitch and roll values and height above bottom was an extremely useful feature.

**MOVEMENT OF *ARGOPECTEN PURPURATUS* AND ITS IMPORTANCE FOR MANAGEMENT.** Wolfgang B. Stotz and Pablo Araya. Departamento de Biología Marina, Facultad de Ciencias del Mar, Universidad Católica del Norte, Sede Coquimbo, Chile.

The Chilean scallop *Argopecten purpuratus* lives attached by byssal threads to stones or shells on the ground at small sizes. At larger sizes and/or when no stones or shells are available, the scallop constructs a shallow hollow on the ground (a “nest”), the upper shell becoming completely covered by sediment thus the individual completely mimicked with the ground. In this situation the scallop is not easily found by an untrained diver and probably

partially hidden to its main predatory crabs. Considering these characteristics of shelter, it is not suspected that this scallop species exhibits much movement on the ground. Nevertheless accumulations of scallops in shallow waters, probably produced by directional active or passive movements of the individuals, have been observed. These accumulations in shallow waters, connected with storms, can cause the stranding of animals. In fact, in 1999 a mass stranding of about 2 million individuals occurred in the bed of Puerto Aldea, which represented a loss of ca. 20% of the entire population of the scallop bed protected and managed by fishers of this village. In order to avoid these losses and to improve the management strategy, the movement of the scallop within the bed was studied. To get an idea of movement intensities within the bed and its eventual directionality, a series of traps were distributed over the area. The traps registered numbers of scallops that moved over a period of time and the direction of their movement. Additionally, with a set of experiments, the influence of the density of scallops in the bed on their movement behavior was studied. Therefore quadrants with different densities of marked individuals were installed and the distance and direction of scallop movement registered. Also the abundance of predators and the losses of scallops caused by predation were registered in these experiments. Only in some sites of the bed the traps registered movement, with no clear predominant direction. These observations suggest that movement occurs actively and is not caused by prevailing bottom currents. The movement was most intense when the density of scallops was high. Coincidentally, with higher densities also higher losses due to predation were registered. The results in general suggest that movement is normally caused by escape reactions to predators. When scallop density gets high, predators find their prey more easily. In this situation also, most scallops when escaping from the predator disturb other scallop individuals, which in turn move, abandoning the shelter of their nest thus becoming more susceptible to predation. Considering these movements of the scallop and its causes and consequences, the maintenance of low densities within the bed is suggested as management strategy to protect the scallops from predation and avoid losses. Individuals in areas of high densities of scallops within the bed, produced by occasional intense recruitments, should be moved by divers to scallop free areas thus increasing production of the bed by an increase of occupied bottom area and not by an increase of its density.

**DEVELOPMENT OF SUSTAINABLE DIVER-FISHERY FOR *PECTEN MAXIMUS* IN NORWAY.** Oivind Strand, Tore Strohmeier and Stein Mortensen. Institute of Marine Research, P.O. Box 1870 Nordnes, N-5817 Bergen, Norway.

The great scallop *Pecten maximus* is distributed along the European Atlantic coasts, north to Lofoten Islands in Norway (69°N). Dredge exploitation of great scallop in Norway has been impeded

by the unfavorable bottom conditions, and scuba diving has been the common harvesting method. A commercial diver-fishery has developed during the last decade, with the main fishing areas west of Trondheim (64°N). The harvest is not regulated, while selling scallops is regulated through licensed dealers. In the beginning of the 1990s this statutory marketing was implemented, and data on catch appeared. Prompted by efforts on developing a shellfish industry the catches increased and has been approximately 400–700 t since 1999, with a value of about 2 million US dollars. In a diver-fishermen team a diver may catch 150–250 kg scallops per day (3–4 scallops per kg), 3–5 days a week. The increase in diver participation in this fishery during 1998 to 2000 incited the Norwegian Labor Inspection Authority to set new requirements on diver certification for scallop harvesters. This reduced the number of diver-fishermen and contributed to regulate the fishing effort. In recent years there are some indications of lower availability of large scallops (>12 cm shell height) and new scallop beds are exploited northward from the main fishing areas. Because the fishery developed the possibility of over-exploitation of the harvestable stock this has been an issue, also among many harvesters. Management of the scallop fishery has also been associated with the development of scallop sea ranching and possibilities of stock enhancement. In 2004 a new law was implemented, regulating stock enhancement through release of cultured sedentary species as mollusks, crustaceans or echinoderms. This law assign the ownership of released scallops within a licensed area. In 2005 the Fishery and Aquaculture Industry Research Fund and Institute of Marine Research initiate a pre-project, involving the diver fishermen, to propose current research priorities and management strategies for a future sustainable scallop diver-fishery.

**PREDATION ON HATCHERY REARED SCALLOP SPAT (*PECTEN MAXIMUS* L.) BY THE BALLAN WRASSE (*LABRUS BERGYLTA*) IN CONTROLLED EXPERIMENTS AND IN SEA RANCHING.** Tore Strohmeier,<sup>1</sup> Guri G. Oppegård<sup>2</sup> and Oivind Strand.<sup>1</sup> <sup>1</sup>Institute of Marine Research, Norway; <sup>2</sup>University of Bergen, Norway.

Sea ranching of scallops (*Pecten maximus*) in Norway has been associated with great losses caused by predation by the edible crab (*Cancer pagurus*). This predation is now hindered by the development of a fence that prevents intrusions of crabs, which has resulted in high survival of scallops in sea ranching. Because crab predation is radically reduced, the opportunity of seeding scallops directly from hatchery to bottom culture emerged. A straightaway release of scallops from hatchery will greatly reduce the labor effort and cost associated with operation of intermediate culture. In the summer of 2002 Helland Skjell AS carried out preliminary experiments with early transfer of spat from intermediate culture to bottom culture. The seeding of scallops from intermediate culture

attracted fish and by seeding 30-mm spat it was observed that the ballan wrasse (*Labrus bergylta*) nab on the scallops. Tank and field experiments were conducted to test if ballan wrasses predate on hatchery reared scallop spat. Ballan wrasses were offered spat from 15 to 35 mm in shell height. Predation was recorded in 20 of 40 tanks and predation was greater than 20% in seven tanks. In these seven tanks the mean predation frequencies significantly decreased with increasing shell height. Predation was not recorded for spat larger than 30 mm in shell height. There was also indication of size dependent predation from the field experiment, but other fishes than ballan wrasse may be the predator.

**SPATIAL AND TEMPORAL DISTRIBUTION OF SEA SCALLOP, *PLACOPECTEN MAGELLANICUS* IN THE BAIE DES CHALEURS, QUEBEC, CANADA.** Benoît Thomas,<sup>1</sup> Michel Giguère<sup>2</sup> and Sylvie Brulotte.<sup>2</sup> <sup>1</sup>Min. Agr. Pêch. Alim., CAMGR, C.P. 340, Grande-Rivière (Québec), G0C 1V0, Canada; <sup>2</sup>Department of Fisheries and Oceans, IML, C.P. 1000, Mont-Joli (Québec), G5H 3Z4, Canada.

The first studies regarding sea scallop spat collecting activities in Gaspésie were done by the private sector in the mid 1980s and by universities in early 1990s in a small part of the peninsula. Between 1999 and 2001, new activities were realized along the coast from Miguasha to Gaspé. Since 2002, more precise factors were examined in the two most successful sectors. Within the last 2 years, the vertical and temporal distributions of the two scallop species (*Placopecten magellanicus* and *Chlamys islandica*) and other associated organisms were examined. In the two sectors of interest, Bays of Tracadigache and Gaspé, a mean of 1200 to 3400 giant scallops/bag was collected in the best sites. The mean number of scallop spat per bag decreased between the autumn and following summer and could be as high as 80% in Tracadigache. The temporal and vertical distribution patterns changed with sector and year so it is still difficult to distinguish a pattern and further analyzes, and trials may be needed in the future. The two sectors are describe as their quality to minimize biofouling and other non productive by catch.

**AGE COMPOSITION AND GROWTH RATES OF QUEEN SCALLOPS *AEQUIPECTEN OPERCULARIS* (L.) AROUND THE ISLE OF MAN.** Belinda J. Vause, Bryce D. Beukers-Stewart and Andrew R. Brand. Port Erin Marine Laboratory, University of Liverpool, Port Erin, Isle of Man, IM9 6JA, British Isles.

Landings of the queen scallop *Aequipecten opercularis* (L.) have fluctuated widely around the Isle of Man since the fishery began in 1969. These fluctuations have been attributed to large variability in recruitment. Regardless it remains the second most

valuable species accounting for nearly 30% of all fish and shellfish landings in 2002. There are no regulations on the fishery, however, it predominantly operates between June and October (the closed season for the great scallop *Pecten maximus* L.) and it is not economically viable to land individuals less than 55 mm in shell height. Stock abundance has been well monitored with fisheries independent stock surveys since 1992, but the age structure and growth rates of the populations have received little attention. The aim of this study is 2-fold. Firstly, to ascertain the best method of age determination and secondly to investigate seasonal and spatial variation in age composition and growth rates of the exploited queen scallops around the Isle of Man. Three methods of determining age composition were used and compared: reading of the annual growth rings from untreated shells, reading of the annual growth rings from treated shells and length frequency analysis. A sample of 150 queen scallops were collected during stock surveys in June and October 2004 from 3 fishing grounds: South PSM, East Douglas and Laxey. An additional sample of 500 queen scallops were collected from South PSM and kept alive in the aquarium. Queen scallops can be difficult to age as their annual growth bands are not always clear. A sub-sample of 150 of the live sample were tagged and aged to the best of our ability in their original state. These animals were then sacrificed and their shells were treated in order to make the annual growth rings more visible. The left (upper) valve was cleaned of epifauna and epiphytes, treated with 5% NaOCl, washed with 98% ethanol, rinsed with water then dried at 60°C for 12 hours. These individuals were then aged again and the results compared to determine if this treatment assisted in the ageing procedure. The remainder of the live samples are being kept in the aquarium for 12 months to validate the formation of an annual growth ring. The 6 frozen samples were treated as earlier mentioned, measured and aged. Ageing the scallops was conducted by three independent readers, before and after treatment, and size was recorded as shell height (mm). Length frequency analysis was conducted using Bhattacharya method on the size data to mathematically determine age composition. The results from these three methods of age determination were compared. It was more difficult and less precise to determine the age of queen scallops before treatment compared to after treatment. An average of 84% (SE 4.6) of untreated shells were considered to be readable by the three readers as opposed to nearly 100% after treatment. In addition, differences between the readers decreased after treatment (precision index before treatment 29.7, after treatment 23.6). A significant difference was found between the ages determined before and after treatment (Kolmogorov-Smirnov test  $Z = -4.206$ ,  $P < 0.001$ ) and regression analysis showed that ageing before treatment was biased towards the lower ages ( $y = 0.77 + 0.54$ ). Further results will be presented comparing length frequency analysis with these results. The age distribution at South PSM was relatively even for the first 3 cohorts with approximately 20%; 1-year olds, 30%; 2-year olds, 30%; 3-years olds, then de-

creasing to 13%; 4-year olds 6%; 5-year olds, as would be expected with natural and fishing mortality. East Douglas also had individuals present in each year class but 3- and 4-year olds were most abundant. At these two grounds there was little seasonal variation in the age compositions of the populations. The age composition at Laxey was dominated by 1-year olds that accounted for 64% in June and 94% in October. In 2003 there was exceptionally high recruitment-population at Laxey, which has survived to produce this strong year class: recruitment at Laxey, prior to this, was poor. Von Bertalanffy growth curves were fitted to the treated shell data, and mean size at age was calculated to investigate seasonal, temporal and spatial variation in growth. Generally mean size at age increased between June and October for 1-, 2- and 3-year olds but not 4- and 5-year olds and at this point the growth curves plateaued. This was expected because most growth takes place over the summer period and growth slows down considerably with age. The difference between mean size of 1-year olds in June and October was 8.3 mm at South PSM, 3.2 mm at East Douglas and 13.6 mm at Laxey. Spatial variation between the three grounds was apparent. In June, Laxey had the smallest 1-year olds of the three grounds, yet the largest 3- and 4-year olds. In contrast, East Douglas had the largest 1-year olds but the smallest 3–5-year olds and South PSM was in between. Despite spatial variation of the size of 1-year olds, the growth curves on the three different grounds intercepted at 2 years old, which is when individuals are approximately 55 mm. Despite the time taken to prepare shells we recommend this method over using live specimens because of the improvements in readability, precision and potentially accuracy (see laboratory experiment). If length frequency analysis is found to give comparable results to prepared shells however, this would be a much more cost effective method because these data can be collected quickly and easily in the field. Differences between the queen scallop age compositions on the different grounds seem to be the product of both variable recruitment and the effects of fishing. Several years of poor recruitment at Laxey has resulted in very low abundances of 2-year olds and older. The increase of 1-year olds in the age composition between June and October may be explained by the increase in size due to summer growth and thus the increase in catch efficiency. Analysis of fishing effort on all grounds around the Isle of Man (from a voluntary logbook scheme) shows that for the season of 2003 Laxey incurred 0%, South PSM 15% and East Douglas 33% of total effort. There was no fishing at Laxey because poor recruitment in previous years resulted in a low abundance of economically viable sized queens. This lack of fishing disturbance may explain the fast growth of these 1-year olds at Laxey, whilst East Douglas had the highest fishing effort and the slowest growing 1-year olds. Fishing pressure may also explain the size distribution of the older cohorts. Fishing gear is selective towards larger individuals so higher effort may drive down mean size at age once a cohort is available to the fishery. This increased knowledge of population dy-



namics and monitoring methods will be used to provide management advice aimed at ensuring the sustainability of the fishery.

# SCALLOP INDUSTRY IN QUEENSLAND—CHANGES AND OPPORTUNITIES. Lew Williams, Assessment & Monitoring Unit, QDPI&F, GPO Box 46, Brisbane Qld Australia, 4001.

In the Queensland scallop industry, based on (*Amusium japonicum balloti*), scallops are primarily harvested by the otter trawl fleet specifically targeting scallop. Lesser amounts of scallop are taken as incidental catch when trawlers target prawn. The scallop year referred to here is from July to June because this allows better description of the changes in annual scallop harvest. Harvest of scallop meat varied between 400 t and 1700 t from 1988/1989 to 2003/2004. The two lowest harvest years occurred in the last 2 years of the series. The number of boats reporting harvest of scallops each year also declined from approximately 300 boats to about 200 boats. At the same time, mean daily harvest per boat for the last 2 years was only slightly lower than the overall mean daily harvest rate. Two major management interventions have been applied to the otter trawl fishery in recent years with flow-on effects to the scallop sector of the fishery. Firstly, otter trawlers now are effectively managed by a “nights fished” quota as well as by other inefficiencies such as time and area closures including some specifically targeted at the scallop sector. In addition, TEDs have been compulsorily introduced into otter trawl nets, which have changed the dynamics of the scallop sector through, for example, the loss of Moreton Bay Bugs as incidental catch making targeted scallop harvest less profitable in some areas. Secondly, known key scallop producing areas have been closed to trawling since 1997 to allow build up of scallop stocks. These strategies have now changed to include a 2-year harvest rotation and changes in size of the areas involved. These management changes have caused changes in the behavior of fishers, because they seek to maximize daily income while taking into account their view of the riskiness of generating that daily income. There is no simple explanation for this dramatic change in the performance of the scallop sector. Some suggest it is the result of the introduction of the otter trawl management plan in 2000, others suggest that it is the effect of specific management measures for the scallop sector; whereas others suggest that there may have been a weather effect due to drought in the adjacent land mass. There has been a flow-on effect into the processing sector (saucer scallops are shucked by hand) especially from Yeppoon to Urangan. The low production in the last couple of years and the change in fishing patterns of the trawlers has caused considerable rationalization in the processing sector with the number of firms available to process scallops declining substantially. These changes have the short term potential to limit the capacity of the

industry to process high levels of harvest when they occur again. Queensland scallop fishery overview available: <http://www.dpi.qld.gov.au/fishweb/12545.html#5>.

# REPRODUCTIVE ECOLOGY OF THE SCALLOP *PECTEN NOVAEZELANDIAE*. James R. Williams<sup>1</sup> and Russell C. Babcock,<sup>2</sup> <sup>1</sup>Leigh Marine Laboratory, University of Auckland, PO Box 349, Warkworth, New Zealand; <sup>2</sup>CSIRO Marine Research, Private Bag No. 5, Wembley 6913, WA, Australia.

Key factors influencing the reproductive success of the scallop *Pecten novaezelandiae* were examined in the Hauraki Gulf, North Island, New Zealand using a combination of field and laboratory studies. Size at maturity was objectively estimated to be 65 mm shell height, although small differences in size at maturity among sites may exist owing to differences in environmental conditions that affect growth rates. Reproductive activity was monitored at four sites from 2000 to 2003 using multiple techniques. Gonad indices appropriate for measuring reproduction were assessed and a method that directly scaled gonad mass to shell height was applied. A visual grading index based on gonad appearance was significantly correlated with quantitative histological and gonad mass data ( $r^2 = 0.74\text{--}0.87$ ). Visual grading facilitated rapid assessment of reproductive condition and could be conducted non-destructively. Repeated observations of visual grade in tagged scallops tethered to the seabed were made *in situ*, the first time that long-term sequential reproductive activity of individuals has been described for a mobile free-spawning animal. Scallops exhibited episodic spawning from spring through to early winter. Temporal variability in gonad size and condition was the result of multiple (serial) spawnings per year. Four to 75% of individuals spawned synchronously between sampling dates. Partial spawnings were common and gonads redeveloped between spawnings. Partial spawning may be part of a bet-hedging strategy to manage the risk of fertilization failure in asynchronously spawning populations. The timing of spawning varied among sites and years, but they often coincided with a sharp drop in sea surface temperature. Scallop spawning behavior reflected their fertilization requirements, releasing millions of gametes in a series of contractions over a short period of time. *In vitro* fertilization success was primarily dependent on sperm concentration, but was also influenced by gamete age and sperm-egg contact time. Maximum fertilization rates (up to 92%) occurred only at high sperm concentrations ( $10^6\text{--}10^7$  sperm  $\text{mL}^{-1}$ ). A polyspermy-adjusted fertilization kinetics model explained 87% of the variation in fertilization success. *Pecten novaezelandiae* gamete traits seem to be adapted to fertilization at high sperm concentration. Consequently, low-density populations may be susceptible to allee effects and reproductive failure.



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## ERRATUM

### **ADDENDUM TO: DIOXIN AND HEAVY-METAL CONTAMINATION OF SHELLFISH AND SEDIMENTS IN ST. LOUIS BAY, MISSISSIPPI AND ADJACENT MARINE WATERS**

**RALPH ELSTON, EDWIN W. CAKE, JR., KAREN HUMPHREY, WAYNE C. ISPHORDING,  
ROD O'CONNOR AND J. E. (JACK) RENSEL**

[Article in *Journal of Shellfish Research*, Vol. 24, No. 4, 1253–1255, 2005.]

Regrettably, following submission of correct page proofs by authors, an error occurred during the final publishing process in the first column of page 1255.

The 2nd line of the left hand column on page 1255 should read “adjacent waters of Mississippi Sound are, on average, about 10%”.

The Publisher greatly regrets the error.



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**COVER PHOTO:** Red urchin (*Strongylocentrotus franciscanus*). Collected from Departure Bay, Nanaimo, BC. Photographed with a Nikon D-70, 105-mm Micro lens with Nikon SS-29 flash unit (lens front mounted). There is a commercial fishery for this species in BC. Photo taken at the Fisheries and Aquaculture Dept facility, Malaspina University-College.

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## REPRODUCTIVE PATTERNS AND THEIR INFLUENCE ON THE POPULATION GENETICS OF SYMPATRIC SPECIES OF THE GENUS *CREPIDULA* (GASTROPODA: CALYPTRAEIDAE)

ALFONSO J. SCHMIDT, JORGE E. TORO<sup>1</sup>\* AND OSCAR R. CHAPARRO

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**ABSTRACT** *Crepidula dilatata* (Lamarck, 1822) and *Crepidula fecunda* (Gallardo, 1979) are sympatric species in southern Chile. Separating them by species is difficult because they are morphologically similar; an important character, which separates them is their comparative larval development. *C. dilatata* includes nutritive eggs within its egg capsules that are consumed by larvae in the capsule during their intracapsular development. Metamorphosis occurs in the capsule, followed by the hatching of crawling juveniles. The *C. fecunda* intracapsular development leads to the release of planktotrophic veliger larvae that persist in the water column for about 15 days prior to settlement and metamorphosis. These two contrasting reproductive strategies (direct and indirect development) may influence the capabilities of these species for dispersal, which could influence their comparative gene flow and population genetics. Newly developing molecular genetic techniques, such as RAPD used in the present study, were useful in identification of the two species studied and provided some initial data on their comparative population genetics. Greater gene flow and interpopulational gene diversity were found in *C. fecunda* (pelagic larva) compared with *C. dilatata* (direct development), with the latter showing populations to be the more genetically heterogeneous within the geographic range studied. It was thus evident that the pattern of larval development (direct or planktonic) influenced the comparative population genetic structure between these two species.

**KEY WORDS:** *Crepidula dilatata*, *C. fecunda*, Chiloé Island, reproductive patterns, RAPD, population genetic

### INTRODUCTION

The basic patterns in reproductive strategies of marine benthic invertebrates include holobenthic cycles, with (a) direct larval development and no pelagic larval phase; (b) completely pelagic cycles and (c) pelagic-benthic cycles where larval development is mixed. The latter case includes the presence of a free-living larva in the water column prior to settlement and metamorphosis in the benthos (Thorson 1950, Mileikovsky 1971, Gallardo 1987, Gallardo 1989).

The presence of a pelagic larva may influence the characteristics of population structure, based on the impact of these stages on the capacity for dispersion of these species (gene flow) (Hunt 1993, Williams & Benzie 1993, Ayre et al. 1997, Hoskin 1997, Kyle & Boulding 2000, Collin 2001, Collin 2003, Nishikawa et al. 2003, Goffredo et al. 2004). A pelagic larva (lecithotrophic or planktotrophic), can remain in the water column for weeks, thus favoring its dispersion, in contrast with the direct mode of development where the individuals hatch from maternal incubatory structures as crawling juveniles (Collin 2001) thus remaining close to their sites of generation. Loss of the larval pelagic phase may favor evolutionary divergence in congeneric species with locally adapted populations; these could diversify in the expression of certain traits in their life histories, reflected in their gene frequencies (Strathmann 1980, Gallardo 1989, Pechenik 1999, Marshall & Keough 2003).

On the other hand, the reproductive strategy, which includes a pelagic larva would guarantee, in the absence of geographic barriers or restrictive oceanic currents, greater larval dispersal and thus greater gene flow. This might however diminish variability in the population structure (Strathmann 1986, Hunt 1993, Palumbi, 1994, Kyle & Boulding 2000, Collin 2001, Hellberg et al. 2002, Whalan et al. 2005), resulting in genetically homogeneous populations.

Species in the Family Calyptraeidae ("slipper limpets") are

typically protandric hermaphrodites. They deposit egg capsules and exercise parental brooding as part of their reproductive strategies. *Crepidula dilatata* (Lamarck 1822) and *Crepidula fecunda* (Gallardo 1979) coexist along a portion of the Chilean coast, having a distribution from 29°58'S to 41°52'S and 12°5'S to 41°31'S, respectively (Collin 2003); both species inhabit both intertidal and subtidal rocky substrates (Gallardo 1977). They can occur at high densities in estuaries and bays along the interior coast of the Island of Chiloé, as well as in protected bays and fjords in the southern channel region of Chile. The species closely resemble each other morphologically, and are best identified based on type of development undergone by their larvae, and secondarily by the maximum size of the adults (Gallardo 1976, 1977, 1979, Brown & Olivares 1996).

Taxonomic studies of the Chilean species of the genus *Crepidula* have been based historically on morphological criteria, where comparisons are made between morphometries of the shell and radula (Gallardo 1979, Brown & Olivares 1996). Also, these species can coexist sympatrically, which makes specific identification even more difficult, particularly when trying to determine the frequency of each species within a given habitat.

Capsules deposited by *C. dilatata* contain nutritive eggs that are consumed as extraembryonic nutrition by larvae developing in the capsules. This factor allows well developed crawling juveniles to hatch from the capsules, having shell lengths of between 0.9 and 1.2 mm (Gallardo 1976, 1977, 1979, Chaparro & Paschke 1990). With *C. fecunda* all the eggs deposited in the capsules develop into veliger larvae that hatch into the water column. They assume a planktotrophic life of about 15 days prior to settlement and metamorphosis, at which time they have a shell length of about  $0.65 \pm 0.028$  mm (Chaparro et al. 2005).

Based on the reproductive mechanisms of these two species it was expected that *C. dilatata* would have a comparatively lower degree of dispersion in the absence of a pelagic larva, consequently reducing gene flow among its populations. On the other hand, *C. fecunda* has a wider dispersion because of its freely drifting larvae, the development of which continues in the water column for a period of about two weeks prior to settlement (Chaparro et al.

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2005). In view of these considerations, the present research was carried out in an attempt to differentiate between these species at the genetic level using the technique of random amplified polymorphic DNA (RAPDs). A comparison was made on the level of dispersion and the degree of influence exercised by the different reproductive strategies of the two species, and their impact on the isolation of populations.

## MATERIALS AND METHODS

### Population Sampling

About 50 specimens of the *Crepidula* species introduced earlier were collected from mixed populations inhabiting the intertidal and subtidal zones at sites along the interior shore of Chiloé Island in southern Chile. The sites sampled included: Quempillén (41°52'S; 73°46'W), Quemchi (42°08'S; 73°28'W), Dalcahue (42°22'S; 73°39'W), Queilen (42°52'S; 73°28'W), Chaiguao (43°08'S; 73°29'W) and Yaldad (43°08'S; 73°44'W) (Fig. 1).

As a means of corroborating later results obtained with molecular markers, limpets from each site were identified with species by examining the type of the larval development occurring in their associated egg capsules.

For the study of molecular markers, a tissue sample of about

1 g was taken from the foot of each specimen, fixed in 95% EtOH, and stored in Eppendorf tubes at -20°C until processed.

### DNA Extraction

Each specimen's DNA was obtained from a subsample of pedal tissue (100–150 mg), which was macerated and digested in lysis-buffer (50 mM Tris-HCL (pH 8.0); 1.0% SDS; 25 mM EDTA) with 200 µg of protease K (Sigma). The samples were incubated at 37°C for 12 h in 1.5 ml Eppendorf tubes; each tube then received 500 µL of a phenol/chloroform/isoamyl alcohol (24:24:1) solution. The mix in each tube was shaken with a vortex mixer for a few seconds and then centrifuged at ×13,000 rpm for 15 min at 5°C. Following this, the supernatant containing the DNA was aspirated, taking care not to touch the intermediate layer. Each supernatant was transferred to a new 1.5 ml (autoclaved) Eppendorf tube. Each extracted DNA sample was resuspended in 200 µL of 95% EtOH and placed in the cold at -18°C for 30 min to precipitate the sample. The EtOH was then removed, leaving the tubes containing the DNA pellet. Concentration and drying of samples was done using a SAVANT DNA model 110 concentrator. Each DNA pellet obtained was resuspended in 200 µL of ultra-distilled water (modified from Toro 1998), and the samples were cleaned with 200 µL of 8M lithium chloride for 3 h at -20°C and then centrifuged at ×10,400 rpm for 30 min at 4°C (Sambrook et al. 2001). The cleanup of the DNA was finalized by eliminating the supernatant and washing the pellet in 70% EtOH. Finally, each pellet was dried for 20 min using the above SAVANT concentrator, resuspended in 200 µL of double-distilled water, and stored at -20°C until amplification.

DNA in the samples was quantified by reading absorbancies at 260 nm in a UV 2120 UV/VIS spectrophotometer. This was carried out following dilution of sample aliquots to 1/20 in distilled water (Sambrook et al. 2001).

### DNA Amplification

Of a total of 49 primers, only three (5'-TAGCCCGCTT-3'; 5'-GTGCGTCCTC-3'; 5'-ACGACGTAGG-3' Biosource International) demonstrated polymorphism.

Amplification of the DNA samples was carried out using the polymerase chain reaction (PCR) employing a total mixture of 25 µL in ultrathin (500 µL) PCR tubes (Gordon Technologies). The reaction mixture was prepared using 1 µL of genomic DNA (ca. 50 ng/µL), 2.5 µL of dNTPs (2 mM), 1.75 µL of MgCl<sub>2</sub> (25 mM), 2.5 µL PCR - buffer ×10, 4.5 µL of the primer (10 µM/µL), 1 unidad of *Taq* ADN polimerasa (5 U/µL) (Invitrogen) and 12.55 µL of double-distilled water (modified from Toro et al. 2004).

The mixture was covered by a drop of mineral oil to avoid evaporation and then each of the samples was placed in a thermo-cycler (Amplifon II, Thermolyne Inc.). The program of the thermo-cycler began with generalized denaturation of the DNA at 95°C for 15 sec followed by 36 cycles, which included a partial denaturation at 94°C for 30 sec, an annealing process at 32°C for 30 sec, and finally a duplication period at 72°C for 1 min; after the 36 cycles the amplified product was maintained at 4°C (Toro et al. 2004).

### Agarose Gel Electrophoresis

All of the DNA fragments amplified with the primers used were separated in agarose gels (Invitrogen Ultra Pure 1000), at 1.2% in TBE 0.5 × buffer (44.5 mM tris base, 44.5 mM boric acid,

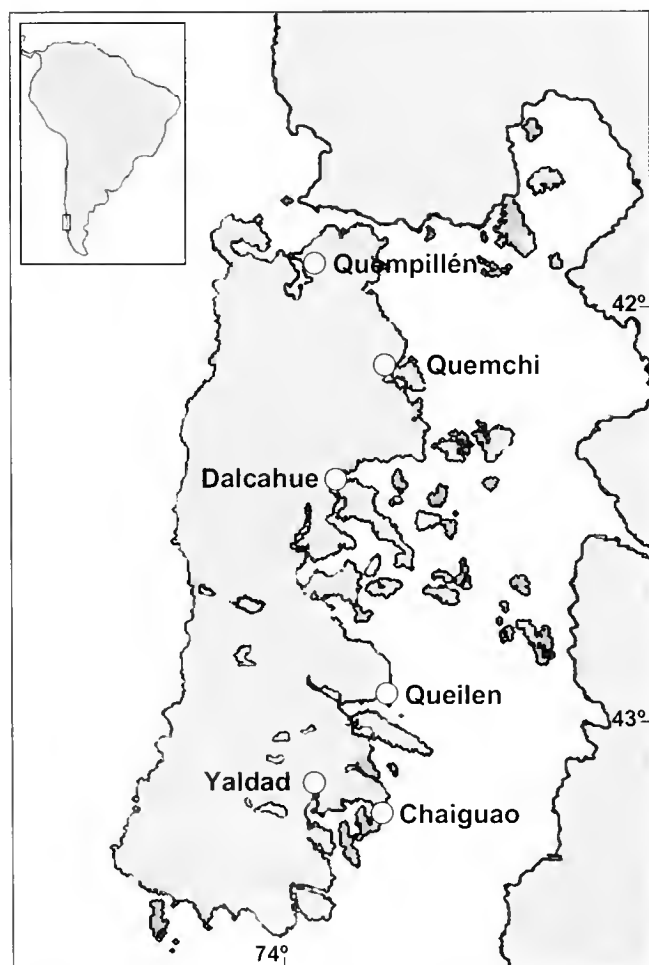


Figure 1. Sampling locations from the eastern coast of Chiloé Island, Southern Chile.

1M EDTA pH 8). Ethidium bromide (10 mg/mL) was added to the gel preparation at a concentration of 1%. Each gel was loaded with a pattern of 1000 bp standard bands (ladder DNA, Promega) for an approximate estimation of the molecular weight of the amplified bands. The gels with the samples incorporated were submerged in an electrophoretic chamber containing TBE 0.5 × buffer (44.5 mM tris base, 44.5 mM boric acid, 1 M EDTA pH 8). Each gel was run at 75 Volts for approximately 1.5 h. Visualization of the band patterns of each gel was done under UV light, and the image of the gel was obtained using a digital camera (modified from Toro 1998).

#### Data Analysis

The results of the RAPDs, were analyzed based on the band patterns recorded by photography of the agarose gels. Codification was made of the bands present (1) and those that were absent (0), which was then configured into a data matrix.

The identification of species was carried out based on the data obtained in the matrix, searching for bands or patterns of bands particular to each of the two species analyzed.

The statistical analysis was carried out using POPGENE 32 computational programs (Population Genetics Software) (Yeh & Boyle 1997) and the GenAlEx version 5.1 program (Peakall & Smouse 2001).

The gene flow ( $N_m$ ) was calculated based on the coefficient of populational differentiation ( $G_{st}$ ) by applying the formula ( $N_m = 0.5 (1 - G_{st})/G_{st}$ ) (Mc. Dermott & Mc Donald 1993). Also determined was the genetic diversity ( $h$ ), and genetic distance ( $D$ ) based on the Nei (1978) formula, and also the number and percentages of polymorphic loci. Based on the pattern of genetic differentiation (genetic distance of Nei 1978), a dendrogram was generated, using the unweighted pair group method with arithmetic mean (UPGMA).

An analysis was carried out, by species, of the number of bands present in each of the populations examined, as well as the mean heterozygosity using the GenAlEx program (Peakall & Smouse 2001). An analysis of molecular variance was also carried out

(AMOVA; Excoffier et al. 1992), to evaluate the inter and intra-populational variation, and estimate the degree of panmixia of the populations (Holmes et al. 2004, Sands et al. 2003, Kruse et al. 2003). Use of a Mantel test (GenAlEx program, version 5.1) allowed for making paired comparisons between genetic distances and geographic distances, using a large number of random permutations. The geographic distances between the sampling sites were approximated by measurement from a nautical chart of the coast-line.

#### RESULTS

The use of the RAPD technique identified 34 loci that were amplified starting with the three primers used.

The patterns of the bands used for each of the species allowed identification of discriminant bands in *Crepidula dilatata* and *Crepidula fecunda* (Fig. 2). The bands existing for *C. fecunda* had weights of about 700, 400 and 300 base pairs. In contrast, *C. dilatata* had bands of only 500 and 300 base pairs. Results from individuals that did not show the presence of discriminant bands were excluded from all analyses because these results may have been caused by technical complications.

Identifications of the samples from all the populations showed that 70% consisted of *C. fecunda* and 19% were *C. dilatata*; 11% of the samples were not identified to species by the molecular method and were eliminated from subsequent analyses (Fig. 3).

The highest percentage of *C. dilatata* occurred at Quempillén, whereas the highest percentages of *C. fecunda* occurred at Dalcahue and Yaldad. The remaining populations (Quemchi, Queilen and Chaiguao) contained both species, with a predominance of *C. fecunda*.

The number of bands amplified for each of the species and each of the populations studied gave an average value slightly lower for *C. dilatata* ( $22 \pm 1.50$ ) than for *C. fecunda* ( $25 \pm 4.67$ ).

At the population level, there was a smaller number of bands recorded for the Quempillén samples, with 18 bands found for *C. fecunda*. The remaining populations maintained equal numbers of bands per species. A few specific bands were identified for indi-

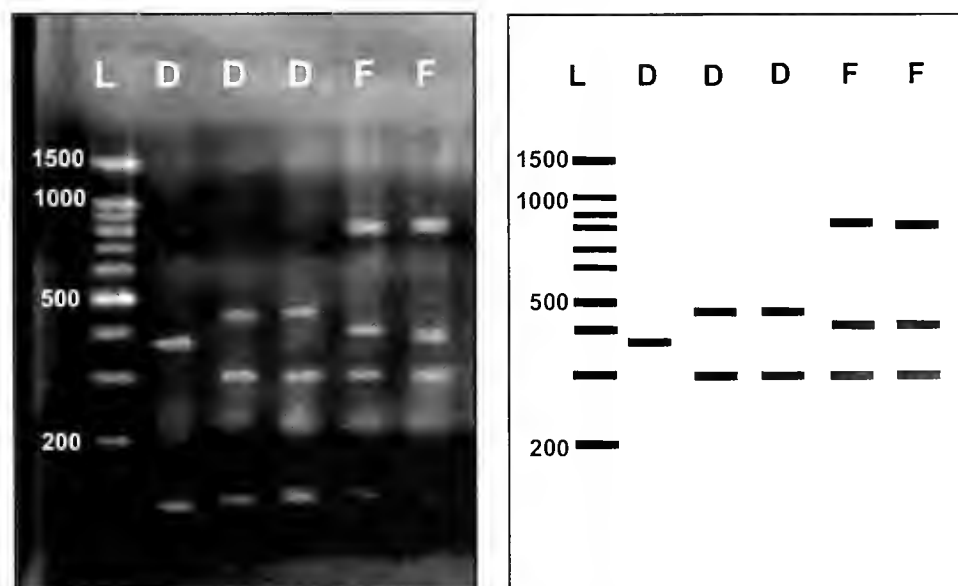


Figure 2. Band patterns resulting from the primer (5'-ACGACGTAGG-3') used to identify species of the genus *Crepidula*. F = *C. fecunda*; D = *C. dilatata* and L = ladder. The band of 380 pb correspond to unidentified specimens.

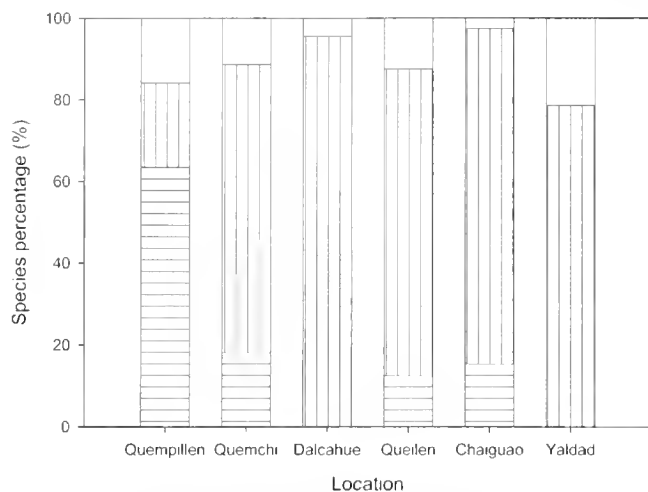


Figure 3. Proportions of *C. dilatata* (vertical lines), *C. fecunda* (horizontal lines) and unidentified specimens (without lines) at each populations sampled along the eastern coast of Chiloé Island.

viduals of *C. dilatata* at Quempillén and Quemchi, which were encountered exclusively in limited populations, suggesting a degree of uniqueness of the limited population.

The gene flow estimated for *C. dilatata* was 0.91 and 1.86 for *C. fecunda*. A paired analysis of gene flow estimated between populations showed that for almost all the locations analyzed, the values were greater for *C. fecunda*; exception to this occurred at Quempillén-Queilen and Quempillén-Chaiguao (Fig. 4).

The intra and interpopulational genetic diversity was greater for *C. fecunda* than for *C. dilatata* at each of the locations sampled, with values of 0.20 and 0.14, respectively for the two species (Table 1). The population at Quempillén had lower values for genetic diversity, both for *C. dilatata* ( $0.09 \pm 0.02$ ) and for *C. fecunda* ( $0.11 \pm 0.03$ ).

In *C. dilatata*, the number of polymorphic loci was nearly constant, fluctuating between 19 and 22, whereas for *C. fecunda*, these fluctuated between 16 and 30; the percentage of polymorphic loci for each species was 85.2% and 100%, respectively. The populations that showed the lowest value for polymorphic loci

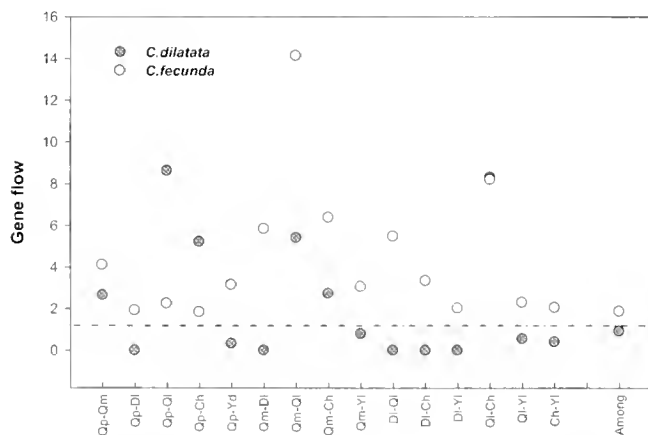


Figure 4. Gene flow of *C. dilatata* and *C. fecunda* species between pairs of populations. Qp, Quempillén; Qm, Quemchi; Dl, Dalcabue; Ql, Queilen; Ch, Chaiguao; Yd, Yaldad; Among, Interspecific analysis for each species.

TABLE 1.  
Genetic population parameters for each sampled areas and species.

Location	Quempillén	Quemchi	Dalcabue	Queilen	Chaiguao	Yaldad	Total
Species							
Heterozygosity							
Mean $\pm$ SD	$0.09 \pm 0.02$	$0.15 \pm 0.03$	$0.16 \pm 0.03$	$0.19 \pm 0.03$	$0.16 \pm 0.03$	$0.16 \pm 0.03$	—
Polymorphic loci (N°)	20	20	27	22	19	29	34
Polymorphic loci (%)	58.8	58.8	79.4	64.7	55.8	85.2	100
Genetic diversity (h) $\pm$ SD	$0.09 \pm 0.14$	$0.15 \pm 0.17$	$0.16 \pm 0.16$	$0.18 \pm 0.14$	$0.14 \pm 0.16$	$0.17 \pm 0.17$	$0.14 \pm 0.14$
Gene Flow (Nm)							1.86
							0.91

*C.d.*, *Crepidula dilatata*; *C.f.*, *Crepidula fecunda*.

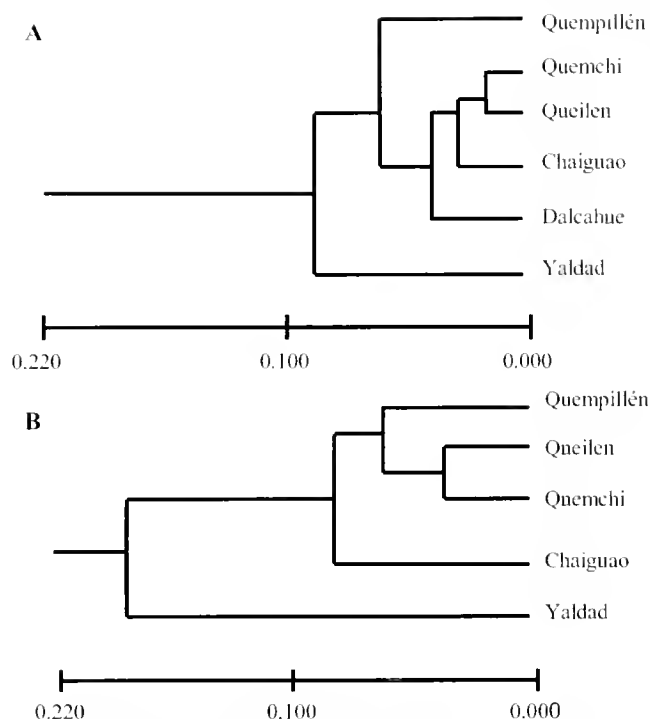


Figure 5. UPGM dendrogram of Nei's genetic distance for (A) *C. fecunda* and (B) *C. dilatata*.

were Chaiguao (19) for *C. dilatata* and Quempillén (16) for *C. fecunda* (Table 1).

For *C. dilatata*, the population at Yaldad was that presenting the greatest genetic distance among all the populations studied, whereas populations at Quempillén, Queilen, Quemchi and Chaiguao showed lower genetic distances. In the case of *C. fecunda*, the populations at Yaldad and Quempillén had the highest values for genetic distance in relation to the other populations studied. The populations at Quemchi, Chaiguao and Dalcabue had lower values for genetic distance, with Dalcabue the most outlayer from the group (Fig. 5, Table 2).

The magnitude of the genetic distance (Nei 1978) for *C. dilatata* was approximately double that for *C. fecunda* (Fig. 5).

The average heterozygosity of each of the populations studied was greater for *C. fecunda* than for *C. dilatata*. In the Quempillén population, *C. dilatata* and *C. fecunda* had lower values than all the other populations studied, suggesting that this location had unusual biological and oceanographic characteristics.

The analysis of molecular variance showed significant differences ( $P < 0.01$ ) between *C. dilatata* and *C. fecunda*, explained by

intrinsic variations in each population. In both species the percentage of interpopulational variation was greater than the intrapopulational variation, indicating that the total genetic variation was, for the most part explained by differences among populations (Table 3).

Correlation between geographic distance and genetic distance, using 5,000 permutations, showed significant correlations ( $P < 0.05$ ) for both species. When the genetic distance was estimated for *C. dilatata* we obtained a Mantel value of  $-0.081$ , whereas the value obtained from the Mantel correlation for *C. fecunda* was  $0.260$ .

## DISCUSSION

The RAPD marker technique permitted identification of discriminant bands, which allowed species discrimination among samples of *Crepidula* spp. as reported for other taxa (Crossland et al. 1993, Andre et al. 1999, Rego et al. 2002, Costa et al. 2004, Liu & Cordes 2004).

The two species were found sympatrically in some of the populations studied. *C. fecunda* was the only species identified from Dalcabue and Yaldad locations (marine conditions), whereas at Quempillén (estuarine conditions) more than 75% of the individuals were *C. dilatata*. This suggested a difference in distribution between the two species based on environmental differences in water mass. The percentage of unidentified individuals (11%), could be because of technical problems encountered during the DNA extraction, as well as in the amplification procedure.

The interpopulational gene flow for *C. dilatata* ( $N_m = 0.91$ ) was less than that for *C. fecunda* ( $N_m = 1.86$ ). Slatkin (1994) indicated that values for this parameter above unity indicated that populations were overcoming the effects of genetic drift, and thus impeding local differentiation.

Paired analyses for populations of the two species, suggested a higher gene flow for *C. fecunda* than for *C. dilatata*. The exceptions that favored the species with direct development (*C. dilatata*) occurred in comparisons between Quempillén and Chaiguao and Quempillén and Queilen. A similar situation has been described for the Chilean oyster *Ostrea chilensis*, where aquaculture activities have distorted the population genetic structure caused by transfer of juveniles from one culture location to another (Toro & Aguila 1996).

The greater degree of gene flow within the populations of *C. dilatata* from Quempillén, Queilen and Chaiguao, could be related to the presence of superficial marine currents (0–30 m), which run from the Gulf of Ancud to the Gulf of Corcovado having a net transport from the north toward the south of Chiloé Island (Silva et al. 1998).

TABLE 2.

Genetic distances for *Crepidula fecunda* (above the diagonal) and *Crepidula dilatata* (under the diagonal) according Nei (1978).

Location	Quempillén	Quemchi	Dalcabue	Queilen	Chaiguao	Yaldad
Quempillén	****	0.0369	0.0805	0.0747	0.0880	0.0734
Quemchi	0.0484	****	0.0336	0.0131	0.0314	0.0670
Dalcabue	—	—	****	0.0367	0.0593	0.1018
Queilen	0.0084	0.0216	—	****	0.0240	0.0976
Chaiguao	0.0178	0.0522	—	0.0067	****	0.1042
Yaldad	0.1700	0.1004	—	0.1650	0.2004	****

TABLE 3.  
Molecular variance (AMOVA) for *C. dilatata* and *C. fecunda* using 999 permutations.

Species	Level	D.f.	Mean Square	% Variance	P	Mode of Development
<i>C. dilatata</i>	Among population	3	14.268	75.5%	<0.01	Direct
	Within population	45	3.365	24.5%		
<i>C. fecunda</i>	Among population	5	41.543	73.9%	<0.01	Planktotrophic
	Within population	171	3.734	26.1%		

This condition could facilitate the drift of juvenile individuals by rafting, as reported for *Crepidula convexa* (Collin 2001).

The greater genetic diversity found for *C. fecunda* in each of the populations studied may be explainable based on its having a pelagic larval stage lasting about two weeks. This amount of time in the waters along the coast of Chiloé would give the larvae of this species ample time for broad dispersion, promoting genetic interchange among populations. The populations of both species at Quempillén showed lower values for genetic diversity, suggesting reduced dispersion, or even larval retention (Bilton et al. 2002). This probability is supported by the hydrographic configuration of the Quempillén estuary, which has natural barriers to the entrance and exit of larvae.

The percentage of polymorphic loci fluctuated between 47.0% and 88.2% for *C. fecunda* and 55.8% to 64.7% for *C. dilatata*. These values were higher than average values for polymorphic loci reported for molluscs (41.2%, Fujio et al. 1983). The percentage of polymorphic loci in the oyster *Ostrea edulis* was between 18.2% and 40.9% (Saavedra et al. 1993), and in the prosobranchs *Cominella lineolata* and *Bedeva hanleyi* it was 24.1% and 41.7% respectively (Hoskin 1997). Based on the preceding, the values presented from the present study are comparatively high, which may be because of the use of DNA based molecular techniques.

The dendrograms for both species show that the genetic distance of *C. fecunda* is half that present in *C. dilatata*. This allows the inference that differences in their reproductive strategies (pelagic larva versus direct development) have an important effect on the genetic structure of the populations of each species. The genetic distances of species with direct development such as *Crepidula cf. convexa* (0.008–0.076) and *Crepidula convexa* (0.037–0.057) (Hoagland 1984) were lower than those presently reported for *C. dilatata* (0.0067–0.2004), which indicates that genetic interchange in the latter species is comparatively less. Greater or lesser genetic interchange may depend on factors such as the population density (Scheltema 1971, Kyle & Boulding 2000), or reproductive variables such as the effective population size (Hedgcock 1994) and presence and duration of the pelagic larval period (Hellberg 1996, Star et al. 2003). *C. convexa* produces from 8–20 capsules per female with each capsule containing 15–25 embryos (Hendler & Franz 1971). *C. dilatata* produces only 9–22 capsules per female with a range of 2–12 embryos per capsule (Penchasadeh et al. 2002). These factors could strongly affect the dispersive capabilities of these species, because a greater number of descendants per generation would be more conducive to net gene flow between populations.

The genetic distance for *C. fecunda* (0.0131–0.1042) was mildly comparable with *Crepidula fornicata* (0.003–0.016) and *Crepidula plana* (0.052–0.097) (Hoagland 1984), all of which have a larval planktonic phase. The low values for genetic distance in *C. fecunda* could be because of local marine currents whose magnitude and direction of flow (Silva et al. 1998) impeded ef-

fective larval transport among some of the populations studied (Scheltema 1971).

The averages of heterozygosity for *C. dilatata* (0.09–0.16) and for *C. fecunda* (0.11–0.19), were within the ranges reported for different species of molluscs (0.05–0.21, Fujio et al. 1983). The average heterozygosity was low for *C. dilatata*, which indicated that the genetic interchange among its populations was very low, suggesting a future decline in the genetic diversity of the gene pool within these populations. This heterozygosity not only may be influenced by the mechanics of reproduction of the species (e.g., absence of pelagic larvae), but also by geographic and oceanographic factors, which may interrupt the flow of individuals among the populations (Palumbi 1994, Collin 2001, Wares et al. 2001, Hellberg et al. 2002, Zacherl et al. 2003, Whalan et al. 2005).

Species with direct development such as *C. convexa* and *C. atrasolea*, share similar characteristics as those of *C. dilatata* based on the results of the AMOVA. In these cases the interpopulational variation was greater than intrapopulation variation, indicating that the genetic variability was greater among the populations (Hellberg 1996). In contrast, the AMOVA data presently obtained for *C. fecunda* do not coincide with previously reported data for *C. fornicata*, as the latter species shows less interpopulational variation (Collin 2001). *C. fecunda*, releases intermediate-sized larvae having shell lengths of 0.18–0.37 mm (Chaparro et al. 2002), whereas *C. fornicata* larvae are 0.44–0.48 mm in length (Collin 2001). Shell length at settlement in these species are  $0.65 \pm 0.028$  mm (Chaparro et al. 2005) and 0.94–1.00 mm (Pechenik et al. 1996), respectively. As cited earlier *C. fecunda* has a pelagic life of only 15 days, whereas that of *C. fornicata* is 34 days (Thouzeau, 1991). Differences in the pelagic period of the life cycle imply different capabilities for larval dispersal, which could have an impact on the genetic homogeneity of the populations (Ayre et al. 1997, Nishikawa et al. 2003).

The positive correlation between geographic and genetic distances, but low values for correlation between populations of *C. fecunda* and *C. dilatata* as shown by the Mantel test, may be because of the fact that the greatest distance between populations was only 230 km, which may influence the sensitivity of the analysis (Hellberg 1996, Exadactylos et al. 2003). Also, the physiographic configuration of the coastline may also have effected the low correlation given by the Mantel test (Wares et al. 2001, Hellberg et al. 2002, Zacherl et al. 2003). For example, whereas the populations at Yaldad and Chaiguao are closer geographically (39 km of coastline), their genetic distances are very high (*C. dilatata* = 0.2004; *C. fecunda* = 0.1042). Yaldad is a protected bay, and is not exposed to direct influence from subantarctic, and estuarine water produced eastward of Chiloé island (Silva et al. 1998). The Chaiguao region is more exposed to direct influences of the former water masses, and we suggest there is very low probability of larvae from Chaiguao entering Yaldad Bay.

To summarize, the present study focused molecular genetic

techniques on the specific differentiation between *C. dilatata* and *C. fecunda*. These methods are a valuable complement to classical morphometric methods, particularly when there exists phenotypic plasticity influenced by environmental conditions at each locality (Seed 1992). The results of the present investigation support the hypothesis that the presence of a pelagic stage in the life cycle of a *Crepidula* species is conducive to comparatively greater inter-population gene flow, which in turn produces greater heterozygosity in the populations. In contrast, direct development leads to an increase in intrapopulation homogeneity and isolation in populations because of constraints in dispersion encountered by the juvenile stages.

Continued research along the lines developed in the present study, supported by ecological and oceanographic studies, is of

great interest because it can provide a more detailed vision of the factors affecting the population genetics of *C. dilatata* and *C. fecunda*. This study is of particular interest in the marine environment because it is not only an interesting, well defined model within a limited geographic area, but it can also serve as a general model of population genetic processes of systematic and evolutionary import by combining classical and newly molecular genetic methods.

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## LOCAL DISTRIBUTION AND TEMPERATURE PREFERENCES OF PREDATORY WHELKS (*THAIS* SPP.) IN TAIWAN: IMPLICATIONS FOR OYSTER CULTURE

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**ABSTRACT** Among the *Thais* species, the broad consensus has long been that *Thais clavigera* (Kuster) is the most noxious predator of cultured oysters in Taiwan. Recently, two new *Thais* species (i.e., *T. rufotincta* Tan & Sigurdsson 1996 and *T. keluo* Tan & Liu 2001) have been identified and named in Taiwanese waters yet their impact on oyster culture is unknown. In this study, the overall impact of the three species on the oyster industry was estimated on the basis of their distribution in the field, their feeding rate and their temperature preference based on laboratory tests. The proportion of *T. clavigera* varied monthly from 24% to 100%, whereas the percentage of *T. keluo* was negatively correlated with low tide levels ( $P < 0.01$ ). *Thais clavigera* occurred widely in the intertidal zone and *T. keluo* and *T. rufotincta* near the subtidal, this distribution pattern was consistent with their specific-preferred temperatures in the upper-limits. As shown in the laboratory, *T. clavigera*, *T. rufotincta* and *T. keluo* preferred 36 °C, 32°C and 30°C water, respectively. In the field, the respective average feeding rate of *T. clavigera*, *T. rufotincta* and *T. keluo* was significantly different at 0.054, 0.010 and 0.038 oysters snail<sup>-1</sup> day<sup>-1</sup> ( $P < 0.05$ ). Based on the abundance of the three *Thais* species at oyster cultural sites (Liu 2002) and their feeding rates, when oyster predation was made up of *T. clavigera*, *T. rufotincta* and *T. keluo*, predation was respectively 87%, 11% and 2%. The most destructive *Thais* species in the oyster industry remains *T. clavigera*, and it accounts for 87% to 100% of all intertidal losses in Taiwan. To the other one-third subtidal culture industry, owing to the use of off-bottom raft or longline method, the distribution of snails extended to subtidal may be limited and the reported major predator is the flatworm of *Stylochus orientalis*.

**KEY WORDS:** *Thais clavigera*, *Thais rufotincta*, *Thais keluo*, temperature preference, distribution, Taiwan

### INTRODUCTION

*Thais clavigera* (Küster) is a common predator in intertidal rocky shores of East Asia, feeding on barnacles, chitons, gastropods, bivalves and polychaetes (Taylor & Morton 1996). It is also a major predator to oyster culture, which is responsible for an annual reduction of up to 10% to 50% of the oyster industry in Taiwan (Lin & Hsu 1979). According to the records of the Taiwan Fisheries Bureau, oyster cultural areas covered 10,700 hectares, and total production was 10,400 metric tons in 2003 (Taiwan Fisheries Bureau 2004). Although oyster predators (i.e., snails of *T. clavigera*, *Cymatium pilcare* [Linnaeus] and *Siphonalia fusoides* [Reeve], crabs of *Scylla serrata* [Forsk.] and *Matuta innaris* [Forsk.], flatworm of *Stylochus orientalis* Bock and fish of *Therapon jarbua* [Forsk.]) have been documented, the prominent two are *T. clavigera* and *S. orientalis* (Kuo 1964, Lin & Tang 1980). Between the two, *S. orientalis* is the major predator in subtidal culture, causing up to 50% of the economic loss (Lin & Tang 1980). On the contrary, *T. clavigera* is the predominant predator intertidally (Kuo et al. 1998).

In light of such predatory behavior, the great economic and ecological significance of the snail *T. clavigera* cannot be denied. However, the situation may have become more complicated because two new *Thais* species have recently been identified, (i.e., *T. rufotincta* [Tan & Sigurdsson 1996], and *T. keluo* [Tan & Liu 2001]), and these two newly named species reportedly occur in the same areas as *T. clavigera*. Not until the present study have we gained much insight into their effects on the oyster industry. In the

present study, therefore, the potential impact of these two congeneric *Thais* predators on the oyster industry of Taiwan was evaluated from the perspective of their field distribution, feeding rate and their temperature preference.

### MATERIALS AND METHODS

Populations of *Thais* species were collected on a monthly basis from September 2000 to January 2002 at oyster cultural sites (i.e., Chiku and Tungkan) and nonoyster cultural sites (i.e., Tamsui and Taichung) (Fig. 1). In a preliminary survey, it was determined that although these three snails inhabit the same areas, by and large, they typically occur in different zones (i.e., *T. clavigera* is common in the intertidal areas, whereas *T. rufotincta* and *T. keluo* are more prevalent in the lower zones). Thus, the decision was made to collect snails in the zones near the subtidal during the periods of low spring tide. Information concerning the tidal heights on the sampling days was obtained from the Central Weather Bureau, Taiwan (Central Weather Bureau 2000–2003). As for data analyses, the number of each *Thais* species (i.e., *T. clavigera*, *T. rufotincta* and *T. keluo*) was counted, and the percentage of each species from every collection was determined.

Feeding experiments were conducted during the November 2000 to October 2001 period. Thirty to thirty-five snails of a single species with a shell length of 20–30 mm were put into an enclosed cage (30 × 30 × 25 cm; mesh size 1.5 cm), and this was replicated 2–4 times for each species. The cages were hung in the oyster cultural area at Chiku. Cultural oysters (about 70–80 individuals cage<sup>-1</sup>) with a shell length of 50–60 mm were provided for the snails to feed *ad libitum*, and fresh oysters were replaced monthly. Additionally, cages containing oysters without snails were also

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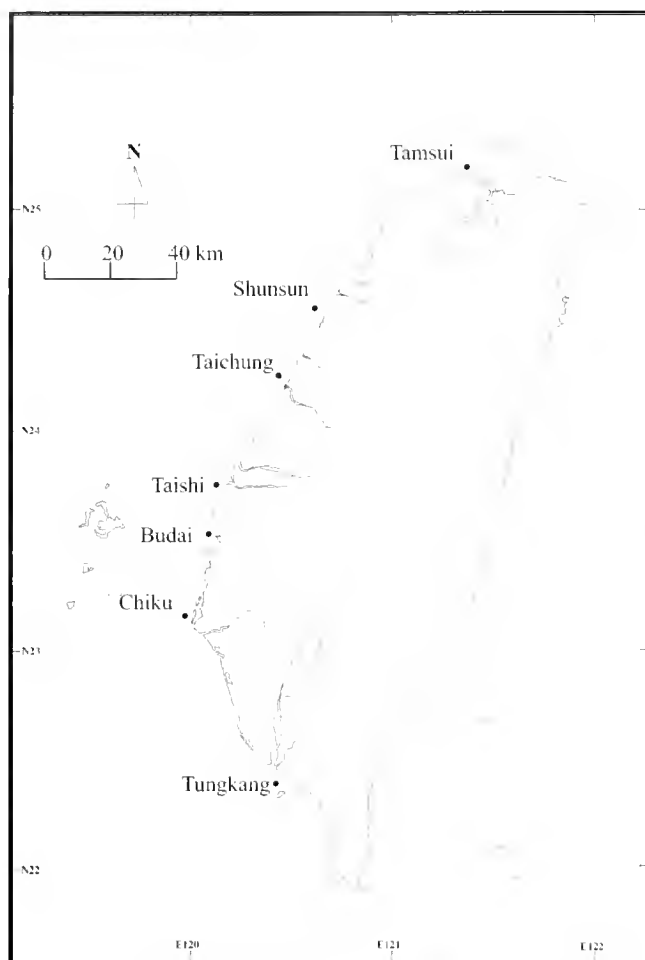


Figure 1. Location map showing the various study sites of the *Thais* species.

supplied as control groups. The number of eaten oysters per snail, the shell lengths and the total wet weights of the snails were calculated to determine the feeding and growth rates.

For the experiments on temperature preference, snails were collected in the zones near the subtidal on the days of low spring tides in July and August 2003 and were kept moist during transportation. For comparative purposes, the collection site Shunsun (Fig. 1), which had enough specimens of the three *Thais* species was sampled. Upon arrival at the laboratory, the snails were kept in their shipping containers until experimental use. The total shipping and handling time prior to the experimental analyses was, at most, 6 h.

A thermo-preferendum chamber was installed horizontally with a 7.14-m long transparent plastic pipe with an inner 11-cm diameter and a 0.5-cm thick wall (Chen & Chen 1991). The pipe was closed at both ends. Two stainless-steel tubes (an outer diameter of 2.1 cm and a wall thickness of 0.05 cm) were placed in the upper one-fifth of the plastic pipe, and these served as heat exchangers. Chilled water was passed through one of the tubes in one direction, whereas heated water was passed through the other in the reverse direction, thereby forming a recirculating countercurrent heat-exchange system. An aeration tube, fitted with a stainless-steel rod to prevent flotation, was installed along the bottom of the plastic pipe to increase dissolved oxygen, improve heat exchange and prevent vertical stratification. On the upper surface of the plastic pipe, seven holes had been drilled to allow us to measure the temperatures and to release test snails into the pipe. When the system was in operation, a continuous thermal gradient was formed inside the pipe, and it ranged from 16°C at one end to 36°C at the other.

Each experiment began with the release of snails into the test pipe in the regions of the low-, mid and high-temperature openings after the thermal gradient had stabilized. The number of each species used in every experiment was in the range of 51–311 individuals. No food was provided during the trials, and none of

TABLE 1.  
*Thais* species sampled at the four sites on the basis of monthly intervals.

	Location							
	Tamsui		Taichung		Chiku		Tungkan	
Date/Species	<i>T. clavigera</i>	<i>T. keluo</i>	<i>T. clavigera</i>	<i>T. keluo</i>	<i>T. clavigera</i>	<i>T. rufotincta</i>	<i>T. rufotincta</i>	<i>T. keluo</i>
Sept. 2000					330 (85%)	60 (15%)		
Oct.					176 (61%)	112 (39%)	355 (98%)	6 (2%)
Nov.					124 (75%)	41 (25%)	814 (100%)	0 (0%)
Dec.	120 (55%)	99 (45%)			490 (87%)	73 (13%)	402 (92%)	37 (8%)
Jan. 2001					74 (97%)	2 (3%)	417 (95%)	20 (5%)
Feb.	131 (95%)	7 (5%)					272 (89%)	35 (11%)
Mar.	134 (89%)	17 (11%)			79 (88%)	11 (12%)	232 (88%)	32 (12%)
Apr.							258 (91%)	26 (9%)
May	62 (50%)	62 (50%)			158 (91%)	15 (9%)	155 (93%)	11 (7%)
June	66 (24%)	211 (76%)			133 (62%)	81 (38%)		
July					657 (89%)	84 (11%)	152 (65%)	83 (35%)
Aug.	182 (97%)	6 (3%)					383 (98%)	7 (2%)
Sept.								
Oct.	85 (89%)	10 (11%)			231 (92%)	20 (8%)	305 (80%)	75 (20%)
Nov.								
Dec.							478 (99%)	6 (1%)
Jan. 2002	76 (93%)	6 (7%)			226 (100%)	1 (0%)		
Aug. 2003 (1)			300 (59%)	210 (41%)				
Aug. (2)			1188 (100%)	0 (0%)				
Sept. 2003			592 (57%)	450 (43%)				

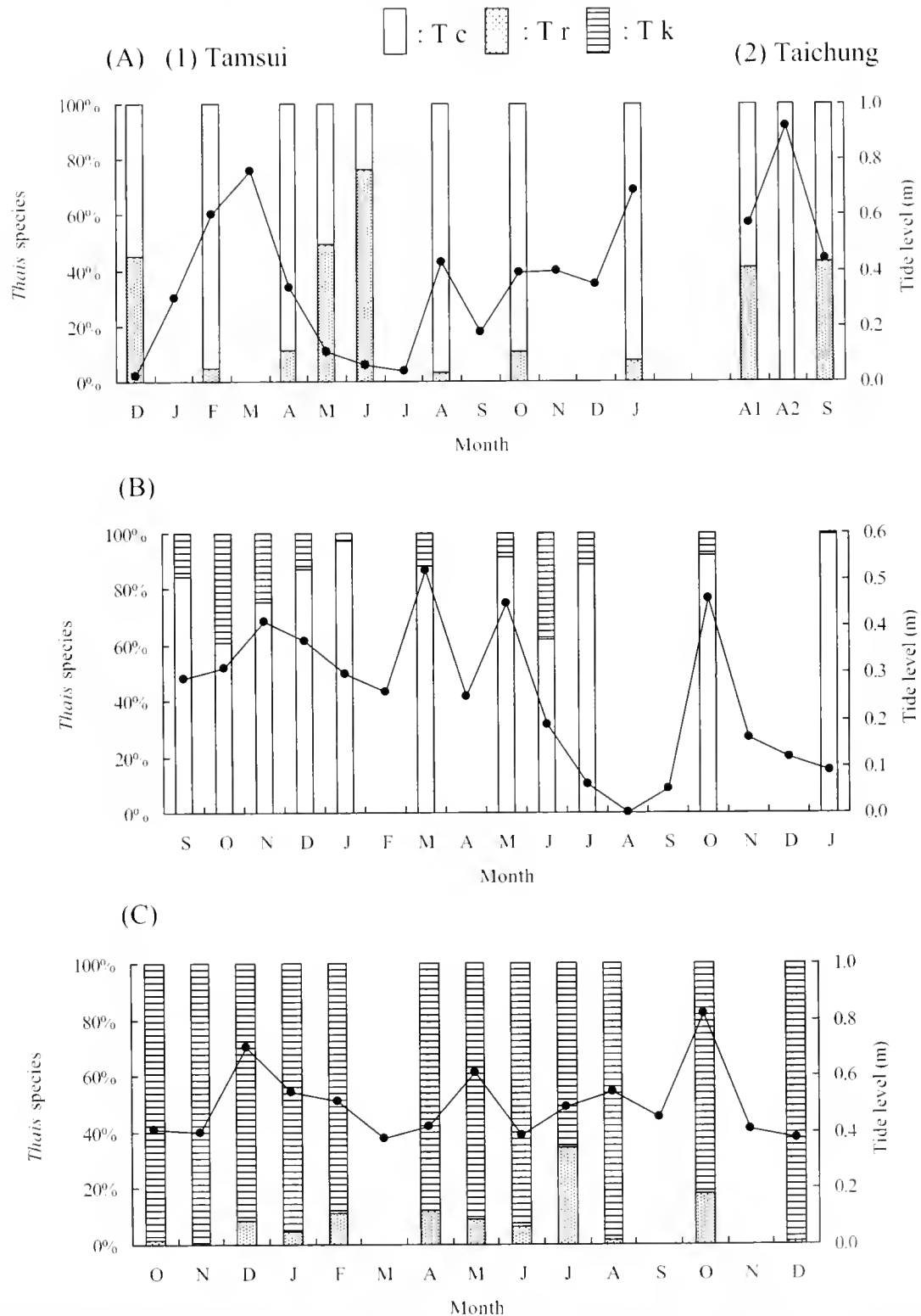


Figure 2. Seasonal variations in the appearance of *Thais* species and tide levels for snails collected from (A) Tamsui and Taichung, (B) Chiku and (C) Tung kang. T c: *T. clavigera*; T r: *T. rufotincta*; T k: *T. keluo*.

the snails that had been used were ever reused. The pipe system and heat-exchange tubes were cleaned prior to each experiment. Because the distribution of snails did not change after the first 24-h during the observation period, over 4 days in the preliminary experiments, the results for the first 24 h were recorded.

For statistical analyses, the simple linear regression was used to determine if species appearance was dependent on tidal level. Linear regressions together with an ANCOVA were applied for the analyses of snails' feeding and growth data. The  $\chi^2$ -test of independence was used to determine whether there were differences in

the 24-h distributions of the thermal preferences of the three *Thais* species.

## RESULTS

Although all the *Thais* species were found at each of the collection sites, one of the three species was relatively uncommon (<1% of the total samples; data not shown) at each site. These were *T. rufotincta* at Tamsui and Taichung, *T. keluo* at Chiku and *T. clavigera* at Tungkan. Because of the limited number of samples, a species that was uncommon at a particular site was excluded in the analysis for that site. In Tamsui, the compositions of *T. clavigera* and *T. keluo* varied monthly from 24% to 97% and 3% to 76% of the total (Table 1 & Fig. 2). A positive correlation between the percentage of *T. clavigera* of the total and low tide was found (i.e.,  $y = 0.43 + 0.936x$ ;  $P < 0.01$ ), but the percentage of *T. keluo* of the total was negatively correlated with the low tide on the sampling days ( $y = 0.568 - 0.932x$ ;  $P < 0.01$ ). In simple terms, the higher the tide level, the less abundant were *T. keluo*. This pattern was similarly observed in the August to September, 2003 collections from Taichung. However, the correlation was not significant for *T. rufotincta* and *T. clavigera* from Chiku or for *T. keluo* and *T. rufotincta* from Tungkan (Fig. 2).

Turning to the feeding experiments, oysters that remained alive during each experimental period were in the ranges of 11% to 90% in *T. clavigera*, 67% to 98% in *T. rufotincta* and 24% to 88% in *T. keluo*, respectively. No dead oysters were found in the control groups. The average feeding rate of *T. clavigera*, *T. rufotincta* and *T. keluo* was respectively 0.054, 0.010 and 0.038 oysters snail<sup>-1</sup> day<sup>-1</sup> (Fig. 3). The slowest feeder was *T. rufotincta* ( $P < 0.05$ ), and monthly variations in the feeding rates of all three species were also apparent ( $P < 0.05$ ) (Table 2). The overall growth of *T. clavigera*, *T. rufotincta* and *T. keluo* was found to have varied respectively from an average of 22.5, 25.2 and 25.3 mm to an average of 40.9, 26.6 and 37.0 mm in length (Fig. 4).

Positive correlations between shell length and cultural interval (month) in all *Thais* species were found (i.e.,  $y = 1.78x + 24.52$ ;  $P < 0.001$ ) for *T. clavigera*;  $y = 0.13x + 24.75$  ( $P < 0.001$ ) for *T. rufotincta*; and  $y = 1.29x + 25.83$  ( $P < 0.001$ ) for *T. keluo*. The slopes significantly differed among species, with a descending order of *T. clavigera* > *T. keluo* > *T. rufotincta* ( $P < 0.05$ ). Additionally, average monthly growth rate differed significantly among species, with values of 0.89, 0.07 and 0.64 mm month<sup>-1</sup> for *T. clavigera*, *T. rufotincta* and *T. keluo*, respectively ( $P < 0.01$ ).

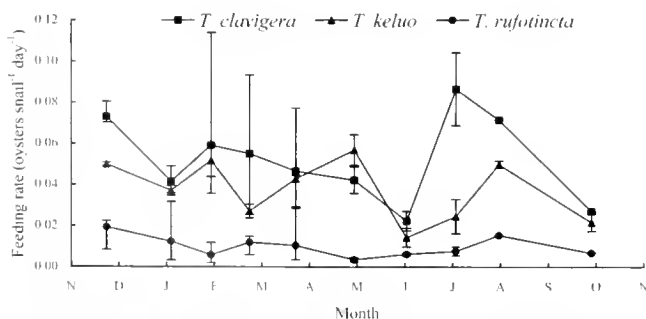


Figure 3. Seasonal changes in the feeding rate of the *Thais* species during the November 2000 to October 2001 period. The symbols represent the median of the feeding rates; the error bars indicate the range of the feeding rates.

TABLE 2.

Tests of the GLM and least square means of the feeding rates of the *Thais* species. The species with different letters indicate significant differences in the feeding rates ( $P < 0.05$ ).

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	29	0.042	0.001	5.25	<0.001
Error	46	0.012	0.000		
Corrected total	75	0.053			

Source	DF	Type 3 SS	Mean Square	F Value	Pr > F
Species	2	0.020	0.010	37.83	<0.001
Month	9	0.006	0.000	2.46	0.022
Species × month	18	0.007	0.000	1.39	0.183

Species	<i>T. clavigera</i>	<i>T. keluo</i>	<i>T. rufotincta</i>
Feeding rate (oysters snail <sup>-1</sup> day <sup>-1</sup> )	0.054 A	0.038 B	0.010 C

With respect to the temperature preference of the three *Thais* species, they were significantly different at all of the collection sites ( $P < 0.01$ ,  $\chi^2$ -test) except at Chiku, the data for which are not shown because *T. clavigera* was the only available species, making comparisons with other species impossible (Fig. 5). Comparatively speaking, the *Thais* species in areas of low- (16°C to 20°C) to mid-temperatures (24°C to 28°C) were inactive during the 24-h experimental period, but what was particularly evident was the distinct tendency for *T. keluo* and *T. rufotincta* to escape areas with high temperatures. Just as in the collection from Shun-sun, the snails in this experiment aggregated in different locations of the high-temperature area, with *T. clavigera*, *T. rufotincta* and *T. keluo* respectively showing a preference for 36°, 32° and 30°C (Fig. 5C).

In summary, the average feeding rates of the snails ranged from 0.010–0.054 oysters snail<sup>-1</sup> day<sup>-1</sup>, or in descending order, *T. clavigera* > *T. keluo* > *T. rufotincta*. Moreover, their respective preferred temperature differed significantly, with *T. keluo* and *T. rufotincta* exhibiting a clear preference for lower temperatures than *T. clavigera*. Such differences in relation to their distribution were, in essence, also indicative of the correlation between the low tide levels of their habitats and the percentage of species that appeared. However, this pattern was not always observed in samples from all the collection sites.

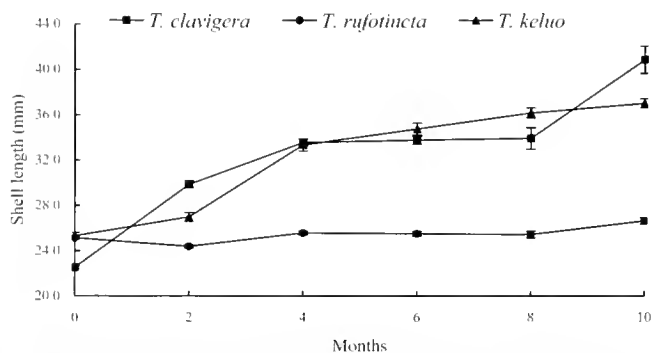


Figure 4. Shell lengths of the *Thais* species at Chiku over the 10-month sampling period. The values are the mean ( $\pm$ SE).

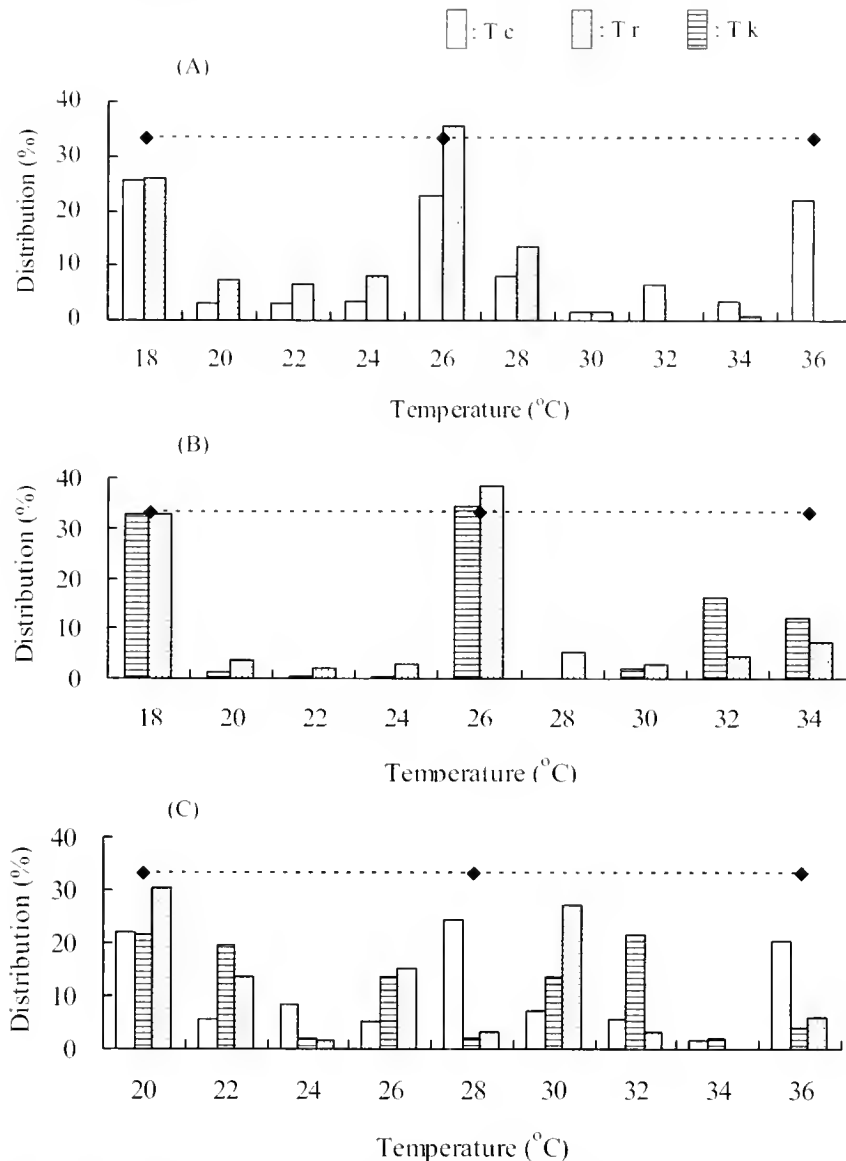


Figure 5. Distribution of *Thais* species along the test pipe in the experiments of temperature preference for snails collected from (A) Tamsui, (B) Tungkang and (C) Shunsun. The difference from each location is significant ( $P < 0.01$ ;  $\chi^2$ -test). T c: *T. clavigera*; T r: *T. rufotincta*; T k: *T. keluo*; -♦- -: Positions of snails' released.

#### DISCUSSION

It has been documented that the distribution of intertidal gastropods reflects the thermal resistance and preferred temperatures of individual species (Newell 1979). Whenever a species had been investigated, the same spatial pattern with respect to other species is found (Underwood 1973). In this study, further evidence in support of spatial zonation was also observed in that *T. clavigera* widely occurred in the intertidal zone, whereas *T. keluo* and *T. rufotincta* appeared lower down. Such distribution pattern was consistent with their preferred temperatures in the upper-limits because *T. keluo* and *T. rufotincta* exhibited lower preferred temperatures than did *T. clavigera* (i.e., 30°, 32° and 36°C), respectively. However, in our results, the correlation between tidal level and species appearance was only found for snails from Tamsui and Taichung but not for those from Chiku or Tungkang (Fig. 2). According to the Central Weather Bureau of Taiwan, the annual average tidal range at the sites of

Tamsui, Taichung, Chiku and Tungkang was respectively 220, 390, 120 and 60 cm (Central Weather Bureau 2000–2003). On the level of thermal and desiccation stress, snails that normally inhabit a small tidal range may be less stressful because of their increased exposure to moisture from wave splash, which allows the lower-down species to distribute upwardly (Newell 1979). Possibly, the marked discrepancy of our results reflects the influence of tidal range on the spatial zonation of these snails.

Depending on the salinity and temperature of the water as well as the size of the oysters or snails, it has been reported earlier that the feeding rates of *T. clavigera* measured in the laboratories ranged from 0.018–0.103 oysters snail<sup>-1</sup> day<sup>-1</sup> (Lin & Hsu 1979). As for the southern oyster drill, *Stramonita haemastoma* (Linnaeus) (Kool 1987) fed on the American oyster *Crassostrea virginica* (Gmelin) ranges from 0.01–0.09 oysters snail<sup>-1</sup> day<sup>-1</sup> (Brown 1997). Our field feeding data were 0.010–0.054 oysters

snail<sup>-1</sup> day<sup>-1</sup>, in other words, falling well within the range reported in previous study. And, there was a 5-fold difference between the lowest and highest feeding rates of the three species, with *T. clavigera* having the highest followed by *T. keluo* (0.038 oysters snail<sup>-1</sup> day<sup>-1</sup>) and *T. rufotincta* (0.010 oysters snail<sup>-1</sup> day<sup>-1</sup>).

Seasonal variations in the abundance of the three *Thais* species have previously been observed at some major cultural sites investigated: Shunsun, Taishi, Budai and Chiku (Fig. 1) (Liu 2002). Densities of *T. clavigera*, *T. rufotincta* and *T. keluo* were in the ranges of 0–272, 1–317 and 0–34 individuals m<sup>-2</sup>, respectively. Among these sites, *T. clavigera* varied in the range of 96% to 100%, 62% to 99%, 64% to 89% and 61% to 99%, respectively. For *T. rufotincta*, it was 0% to 3%, 1% to 38%, 11% to 36% and 1% to 39%, respectively, and for *T. keluo*, it was 0% to 1%, 0%, 0% and 0% to 2%, respectively. Obviously, *T. clavigera* is the most important species simply on the grounds that they always made up more than 60% of the total.

Based on the percent appearance of the three *Thais* species at the intertidal cultural sites and their feeding rates, in the extreme cases of the percent appearance of 61% for *T. clavigera*, 37% for *T. rufotincta* and 2% for *T. keluo*, the impact of oyster predation by *T. clavigera*, *T. rufotincta* and *T. keluo* was 87%, 11% and 2%, respectively. Of course, when *T. clavigera* was the only species, it was responsible for all of the losses. Considering the feeding rate and abundance of the three species, there is no doubt that the most

destructive species to the intertidal oyster industry remains *T. clavigera*. However, intertidal culture is only two-thirds of the whole industry in Taiwan (Kuo et al. 1998).

To the subtidal one-third, *T. rufotincta* and *T. keluo* are the two new potential predators. Formerly, all oysters were cultivated on sandy tidal flats in the western coast of Taiwan and rocks, bamboo or plastic sticks were used to attract oyster spats to settle. Later on, in 1960s, oysters are grown off-bottom by the rack, raft, or longline method and subtidal culture is getting popular (Kuo et al. 1998). Although data on predation by snails in subtidal culture is not available, oyster farmers usually mentioned that the flatworm *S. orientalis* is a serious problem, causing more than 50% of their economic loss (Lin & Tang 1980). Owing to the use of off-bottom raft or longline method, the distribution of snails extends to subtidal may be limited. Consequently, the impact of snail predation on subtidal oyster industry may reduce greatly. However, further field survey on the distribution of these snails in the subtidal is necessary to verify this hypothesis.

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## ENERGY BUDGET OF CULTURED FEMALE ABALONE *HALIOTIS TUBERCULATA* (L.)

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**ABSTRACT** This study evaluates the effects of three diets, at 3 different temperatures (15°C, 18°C and 22°C), on the energy budget of one-year-old abalone. The organisms were fed either an experimental diet based on fishmeal, a commercial abalone diet or fresh seaweed as control. The energy budget was estimated by measuring ingestion, egestion, somatic growth, reproductive investment, respiration, excretion and pedal mucus production. All variables were assessed in female abalone of  $16.36 \pm 0.17$  mm length and  $0.24 \pm 0.03$  g dry weight. Animals fed the formulated diets and cultured at 18°C and 22°C showed better growth than those fed the natural seaweed diet. However, abalone reared at 18°C and fed the formulated diets used 94% of the total energy consumed, with most of this energy allocated to growth, respiration and reproduction. Individuals cultured at 22°C channeled 22% less energy into growth and almost 2.5 times more energy into reproduction than those cultured at 18°C. Hence, diet and temperature were factors that combined to influence growth rates and gonad development in one-year-old *Haliotis tuberculata*.

**KEY WORDS:** cultured abalone, energy budget, formulated diets, *Haliotis tuberculata*.

### INTRODUCTION

The abalone or ormer *Haliotis tuberculata* has been heavily overfished in European waters and considerable interest has therefore arisen in the artificial cultivation of this species (Koike et al. 1979, Hayashi 1980; 1982, Culley 1981, Culley & Peck 1981, La Touche et al. 1993, Mgaya & Mercer 1994, Mgaya & Mercer 1995). Little information is available, however, on energy partitioning in *Haliotis* species (Barkai & Griffiths 1986; 1987; 1988, Donovan & Carefoot 1998, McBride et al. 2001, Gómez-Montes et al. 2002), especially in relation to artificial diets, and data regarding the effect of different macroalgal diets and individuals reared at different temperatures (Emberton 1982, Peck et al. 1987) show variation between the energy ingested and the total energy used.

The study of energy flow in an individual requires an analysis of the partitioning of ingested energy into the major physiological components using the energy budget equation:

$$I - E = Ab - (Pg + Pr + R + U + M)$$

where  $I$  is the energy value of the food ingested,  $E$  is the energy egested in feces,  $Ab$  is the total food ingested minus the total feces egested,  $Pg$  is the energy allocated to production of somatic growth,  $Pr$  is the energy incorporated into the reproductive investment,  $R$  is the energy used for metabolic demands (respiration),  $U$  is the energy value of waste materials, such as ammonia excretion, and  $M$  is the energy value of pedal mucus production (Peck et al. 1987, Davies & Hatcher 1998).

The aim of this study was to estimate the effect of diet and temperature on the energy budget of artificially reared *H. tuberculata*, at a time when the individuals were 13 months old and sexually mature for the first time.

### METHODS

#### Abalone Rearing

The *H. tuberculata* specimens used in this experiment were selected from a previous growth experiment that ran for seven months (López-Acuña 2000). Three females of  $16.36 \pm 0.17$  mm length and  $0.24 \pm 0.03$  g dry weight were used per replicate.

Abalone were fed 1 of the following 3 diets: an experimental diet prepared with fishmeal (FM) as the main protein source (Table 1 and Table 2); a commercial abalone diet (CO), obtained from Gulf Feeds, Australia, and formulated with casein as the main protein source, though the exact formulation of this diet is not available (Table 2); and a seaweed diet (SW) as control, consisting of a fresh mix of 66% red seaweed, *Palmaria palmata*, and 34% green seaweed, *Ulva lactuca* (Table 2). Three replicates per diet were held in natural seawater in constant temperature baths ( $\pm 0.5^\circ\text{C}$ ) at 15°C, 18°C or 22°C. The experimental groups were fed a known amount of food every afternoon and any uneaten food (solid material) was collected the next morning (12 h). Daily rations related to wet body mass were about 1% for dry formulated diets and 5% for fresh seaweed; the dry weight samples of each trial (15°C, 18°C and 22°C) were measured.

#### Determination of the Energy Budget Components

Gross energy content of the samples was estimated by combusting three replicates per treatment in a ballistic bomb calorimeter using benzoic acid as the standard.

#### Absorption (Ab)

Fecal losses were siphoned, placed in a Petri dish, examined under a dissecting microscope to separate any remaining food from solid feces (mucus material) and transferred to a 25-mL centrifuge tube. The samples were then centrifuged at 3000 rpm for 10 min and any excess seawater was dried. Absorption was calculated for each treatment from the total food ingested minus the total feces egested.

#### Respiration (R)

Respiration was determined from the conversion of oxygen uptake ( $\mu\text{L O}_2 \text{ day}^{-1}$ ) to energy loss. A preliminary experiment determined the volume of seawater to be used and the experiment duration at a set temperature. The individuals used were of a specific size ( $16.36 \pm 0.17$  mm and  $0.24 \pm 0.03$  g) and consumed 20% to 40% of the oxygen in no more than two hours. It was difficult to measure oxygen consumption if less than 20% was used, but if more than 40% was consumed the animals were stressed and oxygen consumption was unstable. Organisms were transferred to I-L respirometer vessels at 15°C, 18°C and 22°C.

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TABLE 1.  
Composition of the fishmeal diet (FM).

Ingredients	Percentage of Dry Matter
Fish meal <sup>1</sup>	35.0
Seaweed meal <sup>2</sup>	14.0
Corn meal <sup>3</sup>	10.0
Starch <sup>1</sup>	20.0
Sodium alginate <sup>1</sup>	8.0
Gelatine <sup>1</sup>	4.0
Vitamin mix <sup>4</sup>	1.5
Mineral mix <sup>4</sup>	1.5
Cod oil <sup>1</sup>	6.0

<sup>1</sup> Sigma Chemical Co., UK. <sup>2</sup> *Palmaria palmata* from the English Channel Islands. <sup>3</sup> Maseca, produced in Mexico. <sup>4</sup> Mix based on the requirements for fish in mg (SDS). Vitamins: A, 4.9; B<sub>1</sub>, 1.3; B<sub>2</sub>, 1.6; B<sub>6</sub>, 1.8; B<sub>12</sub>, 0.0015; C, 40; D, 0.30; E, 5.0; H, 0.025; K, 2.25; choline chloride, 75; PABA, 5; folic acid, 0.075; inositol, 5. Minerals: calcium, 1600; cobalt, 0.005; copper, 0.09; iodine, 0.01; ferric citrate, 0.06; potassium, 3.5; selenium, 0.005; zinc, 0.9.

Dissolved oxygen content and temperature were measured in respirometer vessels with and without organisms using a polarographic probe connected to a computer (Endeco, pulsed D.O. Sensor Controller).

#### Ammonia Production (U)

Experimental conditions for ammonia production were the same as those for oxygen consumption. One-liter vessels were filled with seawater and incubated for two hours. Seawater in the incubator tanks was analyzed at the beginning and at the end of each experimental period; three samples from each vessel were used. Total ammonia excretion was calculated as the difference between experimental and blank readings and expressed as  $\mu\text{g-atoms N L}^{-1}$  (Solórzano 1969) from a regression obtained from a series of ammonia standards. Finally, to obtain energy loss by ammonia excretion, a coefficient of  $68.9 \text{ kcal mol}^{-1}$  was used (Brafeld & Solomon 1972).

#### Somatic Growth (Pg)

Somatic growth was assessed as the daily gain in dry body weight measured monthly throughout the growth experiment (L6-

pez-Acuña 2000). In the last month, and after evaluating the energy budget parameters, samples of abalone from each treatment were weighed, using whole body values for those animals that did not develop a gonad at 15°C with the three diets (FM, CO and SW). Animals that did show gonadal development in the treatments at 18°C and 22°C were dissected and the gonad separated from the rest of the body, so that the energy expended on somatic growth could be determined. The energy gain in somatic growth was assessed by combusting the soft tissue from the individuals from each replicate.

#### Reproductive Investment (Pr)

In *Haliotis* species the gonad is closely associated with the digestive gland (the gonadal material includes part of the digestive gland). To obtain a more accurate gonad weight, the digestive gland weight was deducted from the total gonad weight for 25 individuals. The energy consumed was determined as calories per day per animal dry weight, based on the number of days taken to reach the ripe stage (120 days for 18°C and 90 days for 22°C). Gonad weight was then multiplied by its energy content.

With the exception of moisture and lipids, proximate analyses of each diet, feces, whole abalone body from each treatment, whole ripe female gonad and mucus were performed according to standard methods (AOAC 1990). Moisture content was determined by weighing samples before and after drying in a freeze dryer for 36 h at -54°C. Ash content was measured using the remaining dried samples by incinerating at 550°C in a muffle furnace for 8 h. Crude protein content was estimated following the total Kjeldhal nitrogen ( $\text{N} \times 6.25$ ) method. Lipid content was determined by a column procedure using methanol-chloroform-water as the eluting solvent (Bligh & Dyer 1959).

#### Pedal Mucus Production (M)

Pedal mucus production was measured for each experimental trial (FM, CO and SW diets and 15°C, 18°C and 22°C). Each container (400-mL beakers) was cleaned of food and feces, and refilled with UV-filtered seawater. After six hours the animals were removed and each container was rinsed with distilled water to clean off feces and seawater. The mucus from the foot of each abalone was carefully scraped off into the experimental container. The contents of each container were freeze-dried and reweighed to determine the dry weight of each sample.

#### Statistical Analysis

A two-way ANOVA ( $P < 0.05$ ) was applied to determine significant differences among treatment means to compare the parameters of the energy budget between diets and temperatures, followed by comparisons of the means using the Kruskal-Wallis (K-W) test. All statistics were calculated using the Sigma Stat package (2002).

## RESULTS

#### Ingestion, Egestion and Absorption

The energy contents ( $\text{cal g}^{-1}$ ) of whole abalone body and feces from each treatment and of whole ripe female gonad and mucus are summarized in Table 3.

The amount of food consumed increased with temperature. The values obtained for ingestion of the SW diet (control) were significantly different among temperatures:  $25.0 \pm 0.33 \text{ cal day}^{-1}$

TABLE 2.

Proximate composition of the fishmeal (FM), commercial (CO) and seaweed (SW) diets ( $n = 3$ ).

Proximate Composition	Dietary Treatments		
	FM Diet (%)	CO Diet (%)	SW Diet (%)
Dry matter	97.9 $\pm$ 0.14 <sup>a</sup>	98.8 $\pm$ 0.19 <sup>a</sup>	14.0 $\pm$ 0.27 <sup>b</sup>
Crude protein	29.1 $\pm$ 0.22 <sup>b</sup>	33.1 $\pm$ 0.31 <sup>a</sup>	15.5 $\pm$ 0.19 <sup>c</sup>
Total lipid	9.5 $\pm$ 0.09 <sup>b</sup>	11.5 $\pm$ 0.07 <sup>a</sup>	3.1 $\pm$ 0.12 <sup>c</sup>
Ash	16.2 $\pm$ 0.12 <sup>b</sup>	13.5 $\pm$ 0.09 <sup>c</sup>	30.2 $\pm$ 0.14 <sup>a</sup>
NFE + crude fiber <sup>1</sup>	45.1 $\pm$ 0.31 <sup>b</sup>	41.9 $\pm$ 0.26 <sup>c</sup>	51.2 $\pm$ 0.28 <sup>a</sup>
GE <sup>2</sup> (cal g <sup>-1</sup> )	3990 $\pm$ 42 <sup>b</sup>	4400 $\pm$ 89 <sup>a</sup>	2870 $\pm$ 61 <sup>c</sup>
Protein:energy ratio	7.31	7.53	5.41

Values in the same row with different letters are significantly different ( $P < 0.05$ ).

<sup>1</sup> Nitrogen free extract (NFE) + crude fiber =  $100 - (\% \text{ crude protein} + \% \text{ total lipid} + \% \text{ ash})$ .

<sup>2</sup> Gross energy.



TABLE 3.

Gross energy (GE) per gram dry weight of each sample in each treatment: body tissue (BT, minus gonad value), feces, mucus and ripe female gonad of *Haliotis tuberculata*. Mean value  $\pm$  standard error.

Sample	GE* (cal g <sup>-1</sup> )
Fishmeal diet (FM)	
BT reared at 15°C	4001 $\pm$ 55
BT reared at 18°C	4375 $\pm$ 62
BT reared at 22°C	4462 $\pm$ 47
Feces	2127 $\pm$ 63
Commercial diet (CO)	
BT reared at 15°C	3936 $\pm$ 28
BT reared at 18°C	4536 $\pm$ 44
BT reared at 22°C	4542 $\pm$ 68
Feces	2820 $\pm$ 52
Seaweed diet (SW)	
BT reared at 15°C	3652 $\pm$ 30
BT reared at 18°C	3690 $\pm$ 61
BT reared at 22°C	3777 $\pm$ 59
Feces	1699 $\pm$ 55
Ripe female gonad	5688 $\pm$ 64
Mucus (from 25 individuals)	2342 $\pm$ 57

\* Gross energy was calculated burning samples in a ballistic bomb calorimeter.

animal<sup>-1</sup> at 15°C, 33.9  $\pm$  1.94 cal day<sup>-1</sup> animal<sup>-1</sup> at 18°C and 45.8  $\pm$  1.58 cal day<sup>-1</sup> animal<sup>-1</sup> at 22°C (K-W test,  $H = 23.8$ ,  $df = 8$ ,  $P = 0.0025$ ) (Table 4).

The highest energy intake occurred at 18°C and 22°C with values of 70.8  $\pm$  1.82 and 75.8  $\pm$  4.10 cal day<sup>-1</sup> animal<sup>-1</sup> for the FM and CO diets, respectively (Table 4).

The egestion values varied in all treatments and differences among diets and temperatures were therefore observed (K-W test,  $H = 43.1$ ,  $df = 8$ ,  $P = 0.0017$ ). The lowest mean percentage of egested energy was observed in abalone fed the SW diet, which was affected by temperature. A value of 11.2  $\pm$  0.62 cal animal<sup>-1</sup> day<sup>-1</sup> was obtained for abalone fed the CO diet and cultured at 18°C (Table 4).

TABLE 4.

Energy budget (calories per animal per day) of *Haliotis tuberculata*: ingestion (*I*), egestion (*E*), absorption (*Ab*), somatic growth (*Pg*), reproductive investment (*Pr*; null indicates those organisms that did not present gonad development), respiration (*R*), ammonia excretion (*U*) and mucus production (*M*). Mean value  $\pm$  standard error.

°C	Diet	Shell Length (mm)	Body Weight (g)	<i>I</i>	<i>E</i>	<i>Ab</i>	<i>Pg</i>	<i>Pr</i>	<i>R</i>	<i>U</i>	<i>M</i>	Energy Utilized (%)
15°C	FM	16.2 $\pm$ 0.14	0.2 $\pm$ 0.01	29.4 $\pm$ 0.71 <sup>b</sup>	4.2 $\pm$ 0.10 <sup>b</sup>	25.2 $\pm$ 0.6 <sup>a</sup>	10.6 $\pm$ 0.67 <sup>a</sup>	Null	6.3 $\pm$ 0.20 <sup>a</sup>	0.5 $\pm$ 0.02 <sup>a</sup>	0.5 $\pm$ 0.02 <sup>a</sup>	74
	CO	16.7 $\pm$ 0.12	0.2 $\pm$ 0.02	32.1 $\pm$ 1.70 <sup>a</sup>	6.8 $\pm$ 0.13 <sup>a</sup>	25.3 $\pm$ 0.2 <sup>a</sup>	9.7 $\pm$ 0.75 <sup>a</sup>	Null	7.1 $\pm$ 0.21 <sup>a</sup>	0.4 $\pm$ 0.03 <sup>a</sup>	0.5 $\pm$ 0.03 <sup>a</sup>	73
	SW	16.2 $\pm$ 0.11	0.2 $\pm$ 0.01	25.0 $\pm$ 0.33 <sup>b</sup>	3.0 $\pm$ 0.04 <sup>b</sup>	22.1 $\pm$ 0.4 <sup>b</sup>	7.8 $\pm$ 0.69 <sup>b</sup>	Null	3.9 $\pm$ 0.34 <sup>b</sup>	0.4 $\pm$ 0.01 <sup>b</sup>	0.2 $\pm$ 0.01 <sup>a</sup>	60
18°C	FM	16.4 $\pm$ 0.22	0.2 $\pm$ 0.03	70.8 $\pm$ 1.82 <sup>a</sup>	7.9 $\pm$ 0.14 <sup>b</sup>	62.9 $\pm$ 2.3 <sup>a</sup>	39.4 $\pm$ 2.10 <sup>a</sup>	1.2 $\pm$ 0.06 <sup>a</sup>	16.2 $\pm$ 0.5 <sup>a</sup>	0.8 $\pm$ 0.02 <sup>a</sup>	0.7 $\pm$ 0.01 <sup>a</sup>	94
	CO	16.7 $\pm$ 0.36	0.2 $\pm$ 0.02	69.4 $\pm$ 2.40 <sup>a</sup>	11.2 $\pm$ 0.62 <sup>a</sup>	58.2 $\pm$ 1.2 <sup>a</sup>	34.4 $\pm$ 1.77 <sup>a</sup>	1.0 $\pm$ 0.08 <sup>a</sup>	17.1 $\pm$ 0.5 <sup>a</sup>	0.8 $\pm$ 0.01 <sup>a</sup>	0.7 $\pm$ 0.02 <sup>a</sup>	93
	SW	16.3 $\pm$ 0.30	0.2 $\pm$ 0.01	33.9 $\pm$ 1.94 <sup>b</sup>	4.0 $\pm$ 0.51 <sup>b</sup>	29.9 $\pm$ 1.6 <sup>b</sup>	11.3 $\pm$ 0.39 <sup>b</sup>	0.7 $\pm$ 0.04 <sup>b</sup>	5.4 $\pm$ 0.24 <sup>b</sup>	0.5 $\pm$ 0.01 <sup>b</sup>	0.5 $\pm$ 0.01 <sup>a</sup>	64
22°C	FM	16.5 $\pm$ 0.24	0.3 $\pm$ 0.02	68.5 $\pm$ 2.45 <sup>a</sup>	9.0 $\pm$ 0.22 <sup>b</sup>	59.5 $\pm$ 2.8 <sup>a</sup>	25.2 $\pm$ 1.01 <sup>b</sup>	2.3 $\pm$ 0.09 <sup>a</sup>	17.9 $\pm$ 0.7 <sup>a</sup>	1.1 $\pm$ 0.02 <sup>a</sup>	0.9 $\pm$ 0.01 <sup>a</sup>	82
	CO	16.4 $\pm$ 0.20	0.3 $\pm$ 0.02	75.8 $\pm$ 4.10 <sup>a</sup>	10.9 $\pm$ 0.71 <sup>a</sup>	65.9 $\pm$ 2.5 <sup>a</sup>	31.4 $\pm$ 1.23 <sup>a</sup>	2.2 $\pm$ 0.11 <sup>a</sup>	18.4 $\pm$ 0.5 <sup>a</sup>	1.2 $\pm$ 0.03 <sup>a</sup>	0.9 $\pm$ 0.02 <sup>a</sup>	84
	SW	16.4 $\pm$ 0.14	0.2 $\pm$ 0.01	45.8 $\pm$ 1.58 <sup>b</sup>	5.9 $\pm$ 0.16 <sup>c</sup>	40.0 $\pm$ 2.0 <sup>b</sup>	17.1 $\pm$ 0.54 <sup>c</sup>	0.9 $\pm$ 0.03 <sup>b</sup>	8.1 $\pm$ 0.22 <sup>b</sup>	0.7 $\pm$ 0.01 <sup>b</sup>	0.7 $\pm$ 0.01 <sup>a</sup>	71

Fishmeal diet (FM), commercial diet (CO) and seaweed diet (SW), at 15, 18 and 22°C of culture. Energy utilized (%) obtained from 100% *Ab* minus *Pg*, *Pr*, *R*, *U* and *M* (based on the energy budget equation: 1 - *E* = *Ab* - (*Pg* + *Pr* + *R* + *U* + *M*)).

Those individuals fed the SW diet showed a significant increase in absorption with temperature (K-W test,  $H = 30.9$ ,  $df = 8$ ,  $P = 0.002$ ). Energy absorption in abalone fed the FM and CO diets was low at 15°C but consistent at 18°C and 22°C. At 15°C energy absorption was not significantly different between formulated diets (K-W test,  $H = 23.9$ ,  $df = 8$ ,  $P = 0.24$ ) (Table 4).

#### Somatic Growth

The energy values of abalone body tissue (minus gonad value) varied among temperatures for the same diet and among diets at the same temperature (Table 3). The lowest value (3652  $\pm$  30 kcal g<sup>-1</sup>) corresponded to the SW treatment at 15°C and the highest (4542  $\pm$  68 kcal g<sup>-1</sup>) to the CO treatment at 22°C. The energy channeled into somatic growth was calculated by the factors shown in Table 3. Animals fed the formulated diets and grown at 18°C and 22°C (34.4  $\pm$  1.77–39.4  $\pm$  2.10 and 25.2  $\pm$  1.01–31.4  $\pm$  1.23 cal animal<sup>-1</sup> day<sup>-1</sup>, respectively) invested more energy in somatic growth compared with those fed the SW diet at the same temperatures (11.3  $\pm$  0.39–17.1  $\pm$  0.54 cal animal<sup>-1</sup> day<sup>-1</sup>) (Table 4). Furthermore, by the end of the experiment abalone cultured at 18°C and fed the formulated diets expended more energy on growth than those cultured at 22°C with the same diet (Table 4).

#### Reproductive Investment

To convert the energy expended on reproduction, a mean ovary weight of 5688  $\pm$  64 cal g<sup>-1</sup> was used. Females selected from the 15°C treatment showed an indeterminate gonad stage, demonstrating that there was no reproductive investment. Juveniles fed the SW diet and reared at 18°C and 22°C expended the lowest energy on reproduction (0.7  $\pm$  0.04 and 0.9  $\pm$  0.03 cal animal<sup>-1</sup> day<sup>-1</sup>, respectively) (Table 4). When formulated diets were used, the energy expended on reproduction increased to 2.3  $\pm$  0.09 cal animal<sup>-1</sup> day<sup>-1</sup> in the FM treatment at 22°C.

#### Respiration

Metabolic energy loss as a result of respiration was calculated using an oxy-caloric coefficient as follows: 0.00457 cal  $\mu$ lO<sub>2</sub> with NH<sub>3</sub> end product for protein respiration (Crisp 1984), 0.00469 cal  $\mu$ lO<sub>2</sub> for lipid respiration and 0.00504 cal  $\mu$ lO<sub>2</sub> for carbohydrate respiration (Brafeld & Solomon 1972, Elliott & Davidson 1975).

Respiration rates for the organisms fed the FM and CO diets were higher than those for abalone fed the SW diet. Although the size of the animals used was the same for each treatment, the energy consumed was higher in animals cultured at 22°C than in those reared at 15°C (Table 4).

Energy expended through respiration differed between the SW and the formulated diets at 15°C (ANOVA,  $F_{4,18} = 130.5$ ,  $P < 0.0001$ ). At temperatures above 15°C, energy used in respiration increased markedly with temperature. When abalone were maintained at 18°C and 22°C and fed the formulated diets, a significant increment in oxygen consumption was detected, with a mean maximum of  $18.4 \pm 0.5$  cal animal<sup>-1</sup> day<sup>-1</sup> for those cultured at 22°C and fed the CO diet (Table 4).

#### Ammonia Production

Diet and temperature significantly affected the rates of ammonia excretion (K-W test,  $H = 15.7$ ,  $df = 5$ ,  $P \leq 0.001$ ) (Table 4). Individuals fed the formulated diets excreted more ammonia (up to  $1.2 \pm 0.03$  cal animal<sup>-1</sup> day<sup>-1</sup>) than those fed the SW diet in all the temperature treatments. The results show that ammonia production by abalone fed the SW diet was low and that the excretion rates increased from  $0.4 \pm 0.01$  cal animal<sup>-1</sup> day<sup>-1</sup> at 15°C to  $0.7 \pm 0.01$  cal animal<sup>-1</sup> day<sup>-1</sup> at 22°C (Table 4). In general, the rate of ammonia excretion for animals reared at 15°C was 6.06 µg-atoms NH<sub>4</sub> day<sup>-1</sup> on average.

#### Pedal Mucus Production

There were no significant differences in energy content of pedal mucus production among dietary treatments at each experimental temperature (K-W test,  $H = 3.53$ ,  $df = 8$ ,  $P = 0.896$ ). Values were therefore pooled to obtain the energy content of pedal mucus production ( $2342 \pm 57$  kcal g<sup>-1</sup>) to be used in the calculation of the energy budget losses (Table 3). However, a significant effect of temperature on the production of pedal mucus was observed: animals cultured at 18°C and 22°C expended from  $0.7 \pm 0.02$ – $0.9 \pm 0.02$  cal animal<sup>-1</sup> day<sup>-1</sup>, whereas those reared at 15°C spent an average of  $0.5 \pm 0.04$  cal animal<sup>-1</sup> day<sup>-1</sup>. Pedal mucus production showed significant differences when formulated diets were used (ANOVA,  $F_{4,18} = 14.9$ ,  $P < 0.001$ ) (Table 4).

### DISCUSSION

In this study, animals fed the FM diet and cultured at 18°C showed up to 55.7%  $\pm$  3.0% investment in somatic growth from

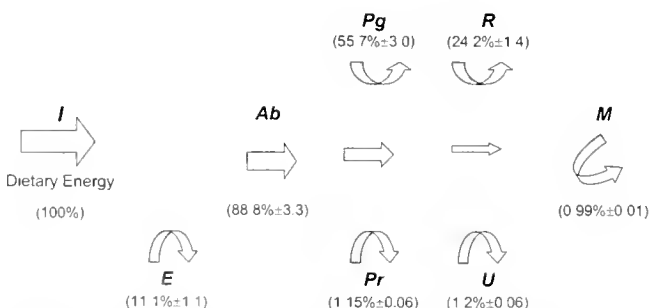


Figure 1. *Haliotis tuberculata* cultured at 18°C and fed a fishmeal-based diet (94% total energy used). Energy flow diagram (calories per animal per day) of ingestion (I), egestion (E), absorption (Ab), somatic growth (Pg), reproductive investment (Pr), respiration (R), ammonia excretion (U) and mucus production (M). Mean value  $\pm$  standard error.

the total energy assimilated (Fig. 1). *Haliotis tuberculata* possesses physiological mechanisms to use energy more efficiently when fed formulated diets and cultured at 18°C than when fed the same diets at 22°C. Peck et al. (1987) reported that the major component of the energy budget was somatic growth (37.5% of total ingestion), and other studies of land-based culture have shown that *H. tuberculata* has maximal growth at temperatures close to 20°C (Shpigel et al. 1996a, Shpigel et al. 1996b).

The daily respiration energy expenditure increased with temperature and diet. Animals fed the formulated diets spent more energy through respiration than those fed seaweed, even at the same temperature. These results show that temperature and diet have an effect on oxygen consumption rates. McBride et al. (2001) also reported that the stress of higher (24°C to 28°C) seawater temperatures increased respiration rates in *H. tuberculata*.

Ammonia excretion is probably influenced by the protein quality of the food offered. Ammonia is assumed to be the dominant end product of protein catabolism in molluscs (Bayne & Newell 1983). When abalone were fed the formulated diets with high protein content (31%) as opposed to the low-protein SW diet (15.5%), ammonia production increased. Therefore, the rates measured may be used to calculate the energy expended by *H. tuberculata* during these experiments and to determine the nitrogen excretion process during the growth stage.

Both respiration and ammonia excretion were affected by temperature and diets. The former was low in abalone fed the SW diet but increased considerably with increasing temperature. The values reported herein are clearly lower than those obtained in other studies using the same species but different diets, culture temperatures and animal sizes: mean energy used by *H. tuberculata*, *H. midae* and *H. kamtschakana* on respiration was 29%, 32% and 59%, respectively and on ammonia excretion, 0.88%, <1% and <1%, respectively, for 50-g abalone (Peck et al. 1987, Barkai & Griffiths 1988, Donovan & Carefoot 1998).

The daily energy expended through mucus production by *H. tuberculata* in this study was from  $0.2 \pm 0.01$ – $0.9 \pm 0.02$  cal g<sup>-1</sup> of the total assimilated energy. Evidence of the influence of temperature on mucus secretion is rare; however, our data show significant effects of temperature on mucus production, with increases at higher temperatures. The same behavior was found for the whelk *Buccinum undatum*, with mean values of 12.4 mg h<sup>-1</sup> at 8.2°C, 13.1 mg h<sup>-1</sup> at 10.5°C and 19.5 mg h<sup>-1</sup> at 15°C (Kideys & Hartnoll 1991).

Peck et al. (1987) found that *H. tuberculata* of 13.7 mm shell length and 0.12 g body dry weight used  $\sim 2.4$  cal day<sup>-1</sup> when fed *Ulva lactuca* and cultured at 15°C, with mucus accounting for  $\sim 23\%$  of the energy consumed. Davies et al. (1990) found  $\sim 23\%$  of consumption was spent on mucus production by *Patella vulgata*. Nevertheless, in this study, animals of the same size fed fresh seaweed and cultured at the same temperature showed an average 3-fold reduction compared with that observed by Peck et al. (1987). These authors demonstrated the important role of mucus in the energy budgets of organisms that use mucus, such as *H. tuberculata*. It is possible that an assessment of the energy used by an individual of the same species can present very different results. Hence, it is important to determine the energy partitioning for a particular organism under specific conditions, and to get a measure of a specific parameter it is necessary that organisms of the same sex, age and body size be compared under the same experimental conditions.

Abalone reared at 18°C and fed the formulated diets used 94% of the total energy consumed, with most of this energy channeled into somatic growth and respiration (Fig. 1). Conversely, individu-

als cultured at 22 °C invested less energy in growth and almost 2.5 times more energy in reproduction than those cultured at 18 °C. In other words, organisms cultured at 18 °C expended more energy on somatic growth and less on reproduction than those reared at 22 °C. Peck et al. (1987) obtained a similar energetic partitioning for abalone cultured at 15 °C and fed fresh seaweed, reporting values of 83% absorption, 15.5% egestion and excretion, 34% growth and 20% respiration, though mucus production was 3-fold higher.

Depending on species, 50-g (dry weight) abalone have been reported to spend 88% (*H. tuberculata*), 74.5% (*H. midae*) and 104% (*H. kamtschatkana*) of the total energy consumed (Peck et al. 1987, Barkai & Griffiths 1988, Donovan & Carefoot 1998). However, abalone in this study cultured under the same conditions (15 °C and fed fresh seaweed) but with a mean dry body weight of only 0.22 g, used ~60% of the energy assimilated. These differences are probably a result of an underestimation of the different variables of the energy budget assessed for abalone in this study. The importance of environmental effects on metabolism and physiology has been demonstrated in aquaculture management

(Brett & Groves 1979). Temperature has been considered one of the most important environmental factors controlling food utilization at all levels and all stages of growth in aquatic poikilothermic animals (Lovell 1989). The data obtained in this study clearly show that temperature and energetically-rich diets have a significant influence on somatic growth and reproductive investment. This information should be of use to aquaculturists and nutritionists in developing a formulated diet and determining the optimal culture conditions for *H. tuberculata*, and it contributes to the scientific knowledge of the associated physiological responses at different culture temperatures.

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## THE HYPOBRANCHIAL GLAND FROM THE PURPLE SNAIL *PLICOPURPURA PANSA* (GOULD, 1853) (PROSOBRANCHIA: MURICIDAE)

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**ABSTRACT** Results are presented on the histology of the hypobranchial gland of the marine muricid *Plicopurpura pansa* (Gould 1853). The general structure and secretory features were investigated using light microscopy and histochemical methods for the determination of tryptophan/indol. The hypobranchial gland of *P. pansa* is an antero-posteriorly elongated organ located on the internal surface area of the mantle, that folds on its posterior side near the rectum, right of the ctenidia and anterior to the kidney. In dead animals it is easily distinguishable by the purple color that develops after removing the shell. Parallel to the hypobranchial gland, in the same position, can be found a black-pigmented structure, presumably the anal gland. The secretory epithelium, which forms the hypobranchial gland consists of at least six different and very long (156.7  $\mu\text{m}$ ) cell types. It was impossible to distinguish clearly the different histological regions of the hypobranchial gland, because the different cell types were uniformly distributed throughout the gland, with the exception of the rectal area. The number of acidophilic granular cells differed markedly between animals, probably because of different levels of secretion. In the mantle cavity was always found a large quantity of mucus and only occasionally acidophilic granulated secretory products. Only the two cell types with acidophilic granules in the hypobranchial gland showed histochemically strong positive reactions for tryptophan, indicating in these cells high concentrations of the precursors for "Tyrian Purple."

**KEY WORDS:** hypobranchial gland, purple snail, *Plicopurpura pansa*, Muricidae

### INTRODUCTION

Most marine snails in the families Muricidae and Thaididae, which make up the genera *Murex*, *Thais* and *Plicopurpura*, produce in the hypobranchial gland (mucus gland) a viscous liquid secretion containing, besides mucus and biologically active compounds, minute amounts of chromogens. These develop enzymatically in light and oxygen into a purple pigment known as "Tyrian Purple," Royal Purple" or "Shellfish Purple." Fretter and Graham (1994) considered the main function of the hypobranchial gland to be a secretor of mucus for trapping and cementing particulate matter sucked into the mantle cavity with the respiratory water current prior to its expulsion.

The carnivorous, gonochoristic, marine, muricid purple snail *Plicopurpura pansa* (Gould, 1853) inhabits intertidal rocky shores exposed to high impact waves of the open sea. The range of *P. pansa* extends from the northwest coast of Mexico (Baja California Sur) (Clench 1947, Keen 1971) to northern Peru (Peña 1970, Paredes et al. 1999). The snail is not too small (shell length averages 30 mm but can be as large as 90 mm), and at low tides it is relatively easily gathered. An exceptional property of *P. pansa*, in comparison with that of other muricids, is that it ejects its dye-producing liquid in such large quantity, that there is no need to kill the animal to obtain the "Tyrian Purple." Furthermore, the hypobranchial gland is so active that the snails can be "milked" periodically without harming the animals (Rios-Jara et al. 1994, Naegel 2005). For these reasons it is not unexpected that *P. pansa* is exploited for "Tyrian Purple" production, probably since pre-Colombian times. In recent years however, with increasing public awareness of natural colors, the commercial exploitation of *P. pansa* for dyeing kimono with "Tyrian Purple" had reached in Mexico such levels as to threaten the survival of the species. In 1988 *P. pansa* had to be declared by the Mexican government a species under special protection (Anonymous, 1988; 1994). De-

spite these exceptional properties of *P. pansa*, first as a source for "Tyrian Purple" and secondly about the state of the endangered snail populations, little is known about the principal life-history features, and many basic biological questions remain. Until now studies are lacking on the histology of the hypobranchial gland of *P. pansa*. The objectives of this study are to gain a better understanding of the biological function of this gland (1) by examining the general morphological features of the *P. pansa* hypobranchial gland using compound light microscopy; (2) determining by histochemical means the inner-cellular sites of tryptophan and (3) comparing the results with previously published reports about the histology of the hypobranchial gland from other Muricidae and Thaididae.

### MATERIALS AND METHODS

More than 100 specimens of an unexploited population of *P. pansa* were collected in 2001 from intertidal rocks on days during extreme low-water spring tides at Playa Cerrito on the Pacific coast (23°19'54"N and 110°10'38"W), about 80 km south-west of La Paz. The average size of the males was 25.87 mm (range 14.86–43.5 mm), of the females 30.14 mm (range 13–73.9 mm).

Injecting a 10% neutral, buffered, formalin solution into the snails preserved the tissue of the animals. About 24 h later in the laboratory eight animals were selected and removed from the shell, subsequently dehydrated in an alcohol series, cleared with butylated hydroxyanisole, and embedded in paraffin. Longitudinal and transversal sections (5  $\mu\text{m}$ ) were prepared of the hypobranchial gland, and according to the methodology by Humason (1979), stained with Ferric Hematoxylin-Eosin or Mallory Trichrome stain. To prove the presence of tryptophan, as the origin of the precursors for "Tyrian Purple" (Verheeken 1989), the histochemical method for the demonstration of tryptophan (Davenport 1960) was applied.

### RESULTS

The hypobranchial gland of *P. pansa* is an antero-posteriorly elongated organ located at the internal surface area of the mantle,

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that folds on its posterior side near the rectum, right of the ctenidia and anterior to the kidney (Fig. 1). On dead animals it is easily distinguishable by the purple color that develops after the removal of the shell. Parallel to the hypobranchial gland in the same position can be found a black pigmented structure, presumably the anal gland.

The secretory epithelium, which forms the hypobranchial gland consists of at least six different and very long (156.7  $\mu\text{m}$ ) nonciliated cell types: (a) eosinophilic cells with an irregular cytoplasmic texture, (b) very abundant goblet mucus cells, (c) cells with strong acidophilic granules, (d) cells with light acidophilic granules, (e) a few cells at the rectal area with very fine basophilic granules and (f) empty cells.

Figure 2 shows a section of the hypobranchial gland: the basal membrane with the longitudinal muscle cells, eosinophilic and goblet mucus cells and cells with acidophilic granules. At the right side of the photograph is shown the mantle cavity with secreted mucus and acidophilic granules. At the left side is shown the external cubical epithelium, which is in contact with the shell.

It was impossible to distinguish clearly the different histological regions of the hypobranchial gland because the different cell types were uniformly distributed in the gland. The exception was the rectal area—the only region where a cell type was found with very fine basophilic granules and where the number of mucus cells and cells with acidophilic granules was reduced. Along the glandular epithelium in the basal tissue were found a few blood vessels and a thin layer of longitudinal smooth muscle fibers.

The number of acidophilic granular cells in the hypobranchial gland differed markedly between different individuals, probably, because of the different stages of secretion. In the mantle cavity there was always a large quantity of mucus (Fig. 3), and only occasionally acidophilic granular secretory products.

Adjacent to the secretory epithelium of the hypobranchial gland is located an acinous glandular-like structure, which could be the rectal or anal gland. The acini are composed of small (10  $\mu\text{m}$ ) nonciliated cubical cells with a large quantity of fine and dark staining basophilic granules. No products of secretion were observed in the lumen of the acini, nor in the duct, which connects to the mantle cavity, the rectum, or the exterior of the snail (Fig. 4).

Only the two cell types with acidophilic granules in the hypo-

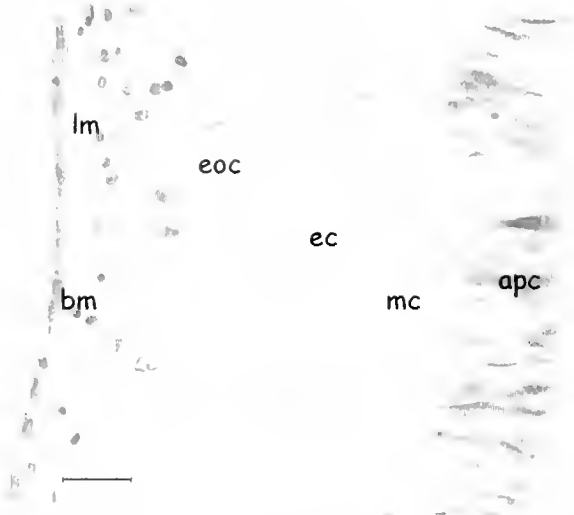


Figure 2. Microphotograph of the hypobranchial gland. bm, basal membrane; ec, empty cell; eoc, eosinophilic granulated cell; apc, acidophilic granules; lm, longitudinal muscle; mc, mucus cell. Bar = 20  $\mu\text{m}$ .

branchial gland showed a histochemically strong positive reaction for tryptophan, indicating that in these cells there are high concentrations of the precursors or chromogens for "Tyrian Purple."

#### DISCUSSION

In the Old World the production and use of "Tyrian purple" was forgotten with the fall of Byzantium (Constantinople) in 1453 A:D. Therefore, it was a big surprise to the scientific community when more than 200 y later, in 1685, William Cole was informed that at the coast of Ireland "Tyrian Purple" from marine snails was still used to mark fine linen (Cole, 1685). After many trials with different snail species at Minehead in England he found the means to obtain the precursors of "Tyrian Purple" from the muricid *Nuccella lapillus*, and the procedure for dyeing materials with them to obtain the final pigment. He made the important observation, first,

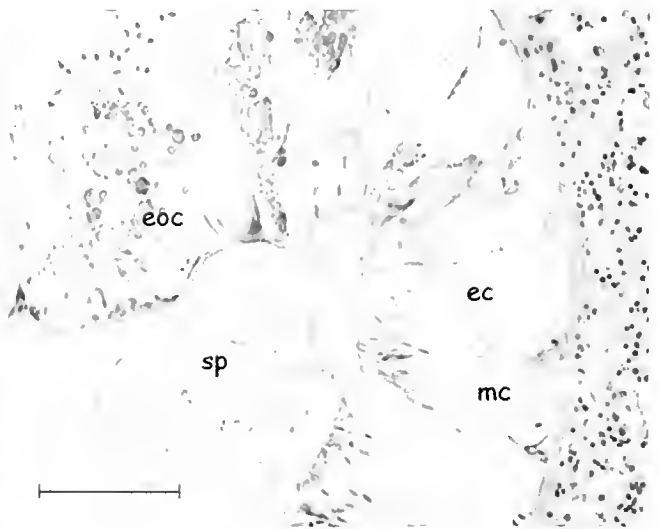


Figure 3. Microphotograph of the hypobranchial gland showing the large quantity of secretory products in the mantle cavity. ec, empty cell; eoc, eosinophilic granulated cell; apc, acidophilic granules; k, kidney; mc, mucus cell; sp, secretory products. Bar = 100  $\mu\text{m}$ .

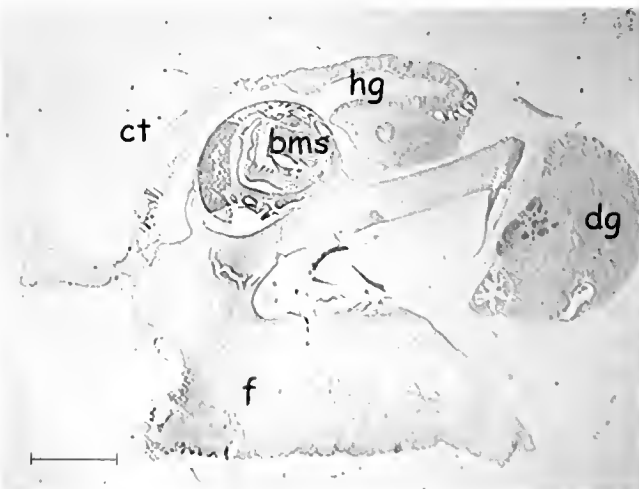


Figure 1. Microphotograph of a section (5  $\mu\text{m}$ ) of the body of *P. pansa*. bms, buccal mass; ct, ctenidium; dg, digestive gland; f, foot; hg, hypobranchial gland. Bar = 1 mm.

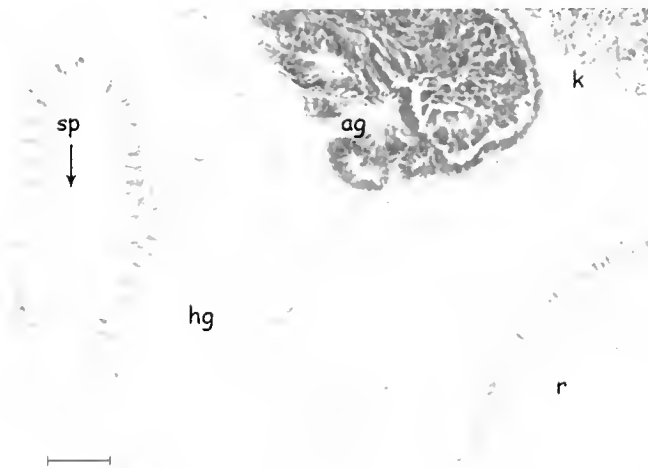


Figure 4. Microphotograph showing the hypobranchial gland, anal gland and rectum. ag, anal gland; hg, hypobranchial gland; k, kidney; sp, secretory products; r, rectum. Bar = 100  $\mu$ m.

that as soon as the colorless fluid is exposed to air and light it becomes immediately yellow and greenish. Soon afterwards it turns into deep emerald green, blue, deep blue, and finally reaches the purple color; and, second, that for this stepwise chemical process light and air are needed. However, 30 y before the discovery by W. Cole about the use of "Tyrian Purple" in Ireland, the English priest Thomas Gage (1655) reported from the New World, Nicoya (Costa Rica), about the commercial exploitation of the secretions of the hypobranchial gland of *P. pansa* to dye garments for rich Spaniards.

Lacaze-Duthiers (1859) showed that the precursors for the pigments are not formed in the kidney or in a vein, as thought during his time, but in a band-like glandular epithelium located at the superior part of the internal mantle cavity. However, because of missing acini and excretory ducts he considered the glandular epithelium not to be a gland. His histological studies of the hypobranchial epithelium of muricids and thaidids raised the interest of many scientists to describe in more detail the histology of the hypobranchial gland.

Letellier (1890) observed in the hypobranchial epithelium of *Purpura lapillus* that the purple producing cells in the middle part of the purple band are much larger, than the neighboring cells. During the same year Bernard (1890) found well-developed innervations in the base of the hypobranchial epithelium of *P. lapillus*, and described that the activity of neuro-epithelial cells has an impact on the production of mucus.

Ersparmer (1946) showed by histochemical methods that in muricids the purple precursors and the enzyme "purpurasi" are localized in the median zone of the hypobranchial body and are kept separate, so that no reaction occurs. Hunt (1973) grouped the many different secretory cell types into two main categories: (a) acid mucin cells and (b) goblet cells. He did not include the purple producing cells. Additionally, as Bernard (1890), he found nerve cells on the base of the gland. Astonishingly, these nerve cells are not in contact with the secretory or purple producing but with ciliated cells (Hunt 1973).

Bolognani-Fantin and Ottaviani (1981) in a histochemical study observed in the hypobranchial gland of *M. brandaris* different cell types: (a) granulated cells with large, and fine granules, (b) with a homogenous cytoplasm, (c) ciliated cells having no impact

on the secretion and (d) empty cells. The different granulated cells, which are only found in the middle area, could reflect different stages of purple production. In another histochemical study of the hypobranchial gland of the marine muricid *Morula granulata* Srilakshmi (1991) described a similar basic arrangement of cells, like described before by Bolognani-Fantin and Ottaviani (1981) and noted the presence of neuro-sensory cells, which form the hypobranchial nerve. Roller et al. (1995) studying by means of light and electron microscopy the hypobranchial gland of the estuarine snail *Stramonita (=Thais) haemastoma canaliculata* defined three anatomically and histologically different areas with eight distinct cell types, which are randomly distributed in the gland. Among these only one is ciliated, a cell type with acidophilic granules. Others are secretory cells that release large amounts of mucus into the mantle cavity.

We observed the same basic types of cells and the great similarity between the previously described hypobranchial glands of different muricids and thaidids. According to Bolognani-Fantin and Ottaviani (1981) the great number of different cells with different functions is a characteristic of the hypobranchial gland of "Tyrian Purple" producing species. Bolognani-Fantin and Ottaviani (1981) mentioned that the hypobranchial gland of a freshwater (*Viviparus viviparus*) and of a terrestrial (*Pomatias elegans*) snail species show a much simpler glandular structure. Bolognani-Fantin and Ottaviani (1981) observed additionally that the cells with fine acidophilic granules react positively to phenolic and indolic substances, which are considered as "Tyrian Purple" precursors. This was confirmed by Srilakshmi (1991) who found strong reactions for tryptophan and tyrosine in the hypobranchial gland. Bolognani-Fantin and Ottaviani (1981) and our results show that only the cells with acidophilic granules react positively to tryptophan. Therefore the acidophilic granulated cells found by Roller et al. (1995) in the hypobranchial gland of the muricid *S. haemastoma canaliculata* could be the purple producing cells.

Lacaze-Duthiers (1859) observed that when the animal contracts vigorously the cells massively open by mechanical or osmotic pressure dispersing their contents into the mantle cavity. Because of the minimal quantity of muscle fibers around the hypobranchial gland of all the earlier mentioned muricids, the likelihood of muscular stimuli is uncertain. Release of the secretion also could be stimulated by neurosecretory activities, because of the presence of neurosensory cells that form the hypobranchial nerve (Srilakshmi 1991). However, there is no evidence of a connection between the nerve and the secretory cells.

Bolognani-Fantin and Ottaviani (1981) mentioned the presence of picrophilic granules on both lateral sites of the hypobranchial gland that reacted with indole, but they could not relate them to the production of mucus or purple. Both authors also mentioned that in the hypobranchial gland of *Murex brandaris* no structural or chemical differences could be observed during the different seasons of the year.

The large number of different cell types and many possible chemical activities in the hypobranchial gland are an indication that the gland has multiple biological functions. These are yet to be discovered.

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## CAN THE COLLECTION OF “TYRIAN PURPLE” FROM *PLICOPURPURA PANSA* (GOULD, 1853) (PROSOBRANCHIA, MURICIDAE) BE BLAMED FOR ITS DECLINING POPULATION?

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**ABSTRACT** Marked, sized and sex determined purple snails *Plicopurpura pansa*, (Gould, 1853) were distributed randomly among other snails in crevices of an intertidal rocky island splashed during high tides by high impact waves. After 89 days 18%, after 117 days 12%, after 145 days 8% and after 183 days only 3% of the marked snails could be recovered. There was no statistically significant difference between size and sex and the recovery rate. In the laboratory we determined the time needed for reattachment to the surface under different situations. Snails placed with the aperture down on a wet surface or in water reattached themselves after about 20 min, snails placed in water on their backs; in about 40 min, and snails left on a wet surface on their backs after 2 hours. After 4 hours only 50% of the snails placed on their backs in a wet surface were found to be reattached. Great differences were noticed in the period needed for reattachment among individual snails. The time needed for the snails to overcome the stress of being detached from the surface and to reattach themselves again can be blamed for the loss of animals during the increasing incoming tides combined with the high impact wave actions. The prohibition of “milking” *P. pansa* to obtain “Tyrian Purple” and to collect the snails as a bait for fishery or as a special food for foreigners should be enforced and should be extended to the removal the snails from the crevices of intertidal rocks.

**KEY WORDS:** purple snail, *Plicopurpura pansa*, muricidae

### INTRODUCTION

In antiquity, the “Tyrian purple” from the Mediterranean muricids, *Murex trunculus*, *M. brandaris* and *Purpura haemastoma* was used extensively to dye materials for the nobility. Because of the enormous number of snails to be killed for the extraction of a minute amount of the chromogens produced by the hypobranchial gland, the scarcity of the snails, and the technical difficulties to obtain the final dye explains why “Tyrian Purple” was at that time a most expensive luxury article. A remarkable exception amongst the muricids is *Plicopurpura pansa* (Gould, 1853) from the Pacific coast of Central America and Mexico, because this mollusc ejects its dye-producing liquid in such a quantity that there is no need to kill the animal to obtain the purple. Furthermore, the dye-producing hypobranchial gland is so active that the snails can be “milked” periodically without harming them (Rios-Jara et al. 1994, Naegel 2005). It is possible that “Tyrian Purple” from *P. pansa* was used on the Pacific coast of Central America and Mexico before Columbus, however our current knowledge about this is very limited. During the Spanish rule, the peninsula of Nicoya in Costa Rica was center for the production of “hilo morado,” which played an important commercial role for the church to charge for their services in “hilo morado,” and for the “gold-hungry” Spanish magistrates to obtain an income from exports to Central and South America. The high demand for “hilo morado” resulted in a decreased population of *P. pansa*, even leading in 1760 to an uprising of the indigenous people in Nicoya against the Spanish administration (Fernández-Guardia 1938, Jinesta 1940). From written reports it is seen that at the end of the 19th century indigenous people from Mexico (Martens, v. 1874) and Nicaragua (Schunck 1880) dyed textiles with “Tyrian Purple” obtained from *P. pansa*. Von Martens mentioned that the high value of purple dyed skirts is explained by the high number of snails needed, which are not numerous at Tehuantepec (Oaxaca) (Martens, v. 1898). In 1909, the ethnologist Zelia Nuttall visited Tehuantepec, and she also reported that the population of the purple snails seemed to be

over-exploited and became scarce, in spite of the careful treatment of the snails during the “ink” collection (Nuttall 1909). In recent years, with the increasing interest in natural colors, the commercial exploitation of the purple snail for dyeing kimonos with natural “Tyrian purple” had reached in Mexico, in the States of Oaxaca, Nayarit, Guerrero and Michoacan, such levels as to threaten the survival of the species (Castillo-Rodríguez & Amezcua-Linares 1992, Acevedo-García 1995). In 1988 *P. pansa* had to be declared a species under special protection by the Mexican government (Anonymous 1988, Anonymous 1994).

Like following a golden thread in the history of the exploitation of *P. pansa* for the production of “Tyrian Purple” it can be observed that it resulted in a decrease of the snail population, in spite of careful treatment of the snails during the “ink” collection and replacement afterwards on the rocks where they have been previously removed. Naegel (2005) has shown that removing the snails from the surface, and Rios-Jara et al. (1994) and Naegel (2005) have demonstrated that periodically collecting the secretion of the hypobranchial gland does not harm the animals. What are the reasons for the declining snail population after “milking”? In a previous study Ramírez-Rodríguez and Naegel (2003) suggested that the snails are just washed away by high impact waves from the rocks after “milking” before being able to attach themselves. In this study this suggestion will be followed up under natural conditions with marked and recaptured snails of different sizes and sexes. Snails reared in the laboratory were used for the determination of the time needed to reattach themselves to the surface after being removed. The results will serve as a prerequisite for the planning, site selection and management of the potential exploitation of *P. pansa* for its “Tyrian purple.”

### MATERIALS AND METHODS

In the laboratory at CICIMAR (La Paz, Baja California Sur, Mexico) from 431 *P. pansa* snails the size and sex were determined and afterwards they were tagged with colored and numbered marks as used in bee-keeping. On November 11, 2005 the snails (266 males with an average total shell length of 27.9 mm; range 17.5–44.4 mm and 165 females with an average total shell

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TABLE 1.

Size ranges of marked male and female of *P. pansa* snails distributed on November 11, 2004 at Playa Cerritos, Baja California Sur, Mexico.

Size (mm)	Males		Females	
	N	SD	n	SD
17.5–26.4	90	1.78	40	1.99
26.5–35.4	165	2.46	88	2.32
35.5–47.4	11	2.77	37	2.61
Total	266	4.04	165	5.54

SD = Standard deviation

length of 30.36 mm; range 18.2–47.4) (Table 1) were transferred to Playa Cerritos (23°19'49"N, 110°10'45"W) (Fig. 1) about 120 km southwest of La Paz, Baja California Sur, Mexico and placed randomly amongst other *P. pansa* snails in crevices on an intertidal rocky island. At low tide the basalt rock covers an area of about 800 m<sup>2</sup>, from which, even at low tides, are accessible only about 400 m<sup>2</sup> because of the high impact waves striking the rock. The island has several elevated points, the highest with 3.04 m above the mean tide water level, with many crevices located opposite the impact of the waves. We determined in a rough estimate that only about 1/8th of the total rock area is suited as a habitat for *P. pansa*.

The island was revisited during extreme low tides after 89, 117, 145 and 183 days, the marked animals recaptured, and afterwards placed carefully amongst other snails in crevices. The number and size of the recaptured marked snails was examined to determine possible effects of size and sex on the rate of recovery, and  $\chi^2$  test

applied to determine significant differences. A level of significance of  $P < 0.05$  was applied.

In the laboratory we determined the time needed for snails of different sizes ( $n = 382$ , average size: 32.2 mm, size range: 19–46.1 mm, SD  $\pm 4$  mm) and sex (males:  $n = 96$ , average size: 32.2 mm, size range: 24.3–44 mm, SD  $\pm 3.8$  mm; females:  $n = 96$ , average size 33.5 mm, size range: 23.1–46.1 mm, SD  $\pm 4.6$  mm) to reattach. For each position of the snails we repeated the experiments four times. We used a transparent plastic tray with a 5-cm wall height to observe the attachment of the foot of the snails to the surface. In the experiments with snails lying on their backs on the wet surface and after 4 hours remaining unattached to the surface we had to sprinkle a few drops of seawater on the operculum to prevent dehydration of the animals. To determine whether possible differences exist between laboratory and field snails in the time for reattachment required, we performed two additional experiments at Playa Cerritos with snails ( $n = 48$ ; average size: 28.7 mm; size range: 22–36.9 mm; SD  $\pm 3.8$  mm).

The differences in the time needed for reattachment to the surface in the field and in the laboratory among the different size classes and sex were determined by 1-way ANOVA. The Tukey test was further used to determine significant differences and  $P < 0.05$  was used as the significance level.

## RESULTS

On November 11, 2004, during an extreme low tide (tidal difference between low and high tide: 1.89 m), 431 marked snails were placed randomly amongst other *P. pansa* snails in crevices of the intertidal rocky island.

After 89 days (difference between low and high tide of more than 2 m) 77 marked snails were recovered (57 males and 20 females), after 117 days 52 animals (19 females, 33 males), after 145 days 36 snails (12 females and 24 males) and after 183 days (difference between low and high tide: 1.30 m) 13 animals (no females and 13 males). To determine if the size of the animals had an impact on the likelihood of recovery, the recovered snails were divided into three size classes. Table 2 shows in three size classes the number of recovered females and males during the experimental period. After 89 days from the size class 17.5–26.4 mm 27% of the animals were recovered. From the size class 26.5–35.4 mm 14% of the snails were recovered. From the larger size class (35.5–47.4 mm), after 117 days, not one male and after 145 days only one female was recovered. After 183 days of the experiment not one female was recovered from all three size classes, and from the male snails 7% from the smallest and only 3% from the medium size group. No statistical differences were found among the three size classes.

In the laboratory we determined the time needed for reattachment of the snails to the surface. Great differences were observed between individual snails and the time needed for their reattachment. These individual differences could not be statistically related to the size or sex of the animals ( $P > 0.05$ ). For animals placed on their backs on a wet surface, it took an average of more than 2 hours for reattachment (Fig. 2). Seventy percent of the snails placed with the aperture down on the wet surface, or in water, reattached themselves on the surface only after 20 min, and 90% of the snails were reattached after 1 h (Fig. 3). There was no statistic difference ( $P > 0.05$ ) in the time needed for reattachment between snails placed on the aperture in water and on a wet surface. Animals placed on their backs on the wet surface and in water behaved differently. A statistical difference ( $P < 0.05$ ) was found

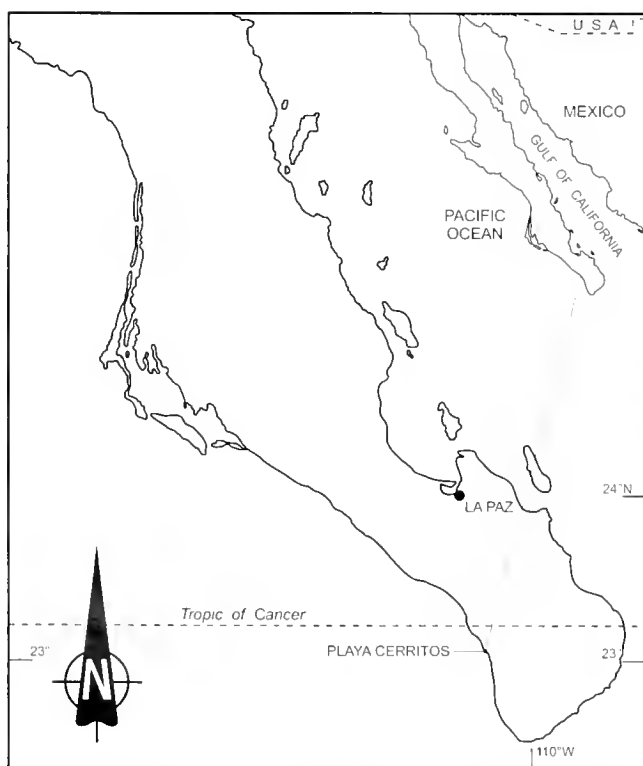


Figure 1. Location of Playa Cerritos and of La Paz, Baja California Sur, Mexico.

TABLE 2.

Size classes of *P. pansa* snails recaptured at Playa Cerritos, Baja California Sur, Mexico, after 89, 117, 145 and 183 days.

Size (mm)	89 Days		117 Days		145 Days		183 Days	
	Number	%	Number	%	Number	%	Number	%
17.5–26.4	35	27%	15	12%	10	8%	7	5%
26.5–35.4	36	14%	34	13%	25	10%	6	2%
35.5–44.4	6	13%	3	6%	1	2%	0	0%
Total	77	18%	52	12%	36	8%	13	3%

among animals placed their backs on the wet surface and the other three groups. After 2 hours, 80% of the snails on their backs in water turned over on their aperture and attached themselves to the surface, and after 6 hours 86% were attaching. From the 96 snails placed on their backs on the wet surface, only 50% were attached after 4 hours. After 4 hours the remaining 48 snails received a few drops of seawater on the operculum and the animals revived, and after 6 hours 86% of the snails were found attached to the surface (Fig. 3). This result shows the danger, for snails lying for longer periods in the dry on their backs, of being desiccated (Fig. 3).

We determined at Playa Cerritos the time needed for reattachment of snails placed with the aperture down and in water. The average time for reattachment took 11.7 min (SD  $\pm$  13.5 min). A result, which is statistically not different ( $P < 0.05$ ) to the findings from the laboratory tests described before.

#### DISCUSSION

The recapture rate of snails placed carefully into crevices was astonishingly low. After 89 days of the 431 snails only 18% were recovered, mainly (27%) from the smallest size class. It is more likely for large and not yet attached animals to be swept away by strong waves. After 183 days of the experiment only 3% of the animals (13) were recovered. Wave-swept shores, like the intertidal rocks at Playa Cerritos, are physically harsh environments, and only crevices give the snails protection from not being washed away by high impact waves during high tides. *P. pansa* is a most spatially restricted species and over 75% is found primarily in crevices (Garrity, 1984). This microhabitat is important in reducing temperature and water loss. *P. pansa* is extremely sensitive to

heat and desiccation on open surfaces. Animals removed from the crevices during daytime low tides reach higher tissue temperatures, lose more mass and suffer higher mortalities than control animals (Garrity 1984). Removing the snails carefully from the surface and replacing them afterwards in water does not cause mortalities (Naegel 2005). This finding is in contrast to the report by Castillo-Rodríguez and Amezcua-Linares (1992) who blamed the mortalities on the brusque removal of snails from the crevices, and the bending of the operculum and the muscular foot for the extraction of the dye precursors. Acevedo-García (1995) mentioned differences in the way commercial "ink-collectors" and traditional indigenous people remove the snails from the crevices in the rocks: not to harm the animals the indigenous people use a wooden stick in the form of a spatula, the "ink-collectors," however, use a hook made out of wire, which sometimes breaks the shell. Acevedo-García (1995) also mentioned the different methods used by indigenous people and commercial "ink-collectors" to obtain the dye secretion. To stimulate the expulsion of the "ink" the indigenous people spit and/or blow on the operculum, in contrast to the commercial "ink-collectors" who press the operculum with the thumb, which causes a weakening of the fixation of the animals to the rocks, from where they can easily be washed away by the strong waves. Some "ink collectors" place the snails after "milking" together with their "milk" in a small pot. The narcotizing compounds in the "milk" reduce the capability of the snails, after their release, to attach themselves fast enough, and consequently are in danger of being washed away.

During our field experiments we didn't use a spatula or a hook to remove the snails from the crevices so as not to hurt them. We replaced carefully the snails again in crevices. Despite all this care the recovery rate was very low.

The removal and the "milking" of the purple snails cannot

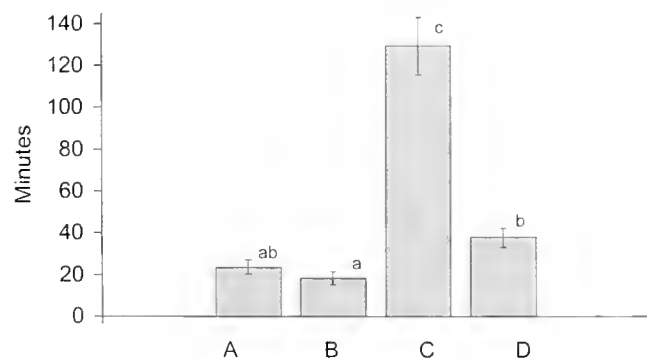


Figure 2. Average time needed by *P. pansa* snails to reattach themselves after removal to the surface. A = Aperture down on a wet surface ( $n = 94$ ), B = Aperture down in water ( $n = 96$ ), C = Snails on the back of the shell on a wet surface ( $n = 96$ ), D = Snails on the back of the shell in water ( $n = 96$ ). Columns with different superscripts are statistically significantly different ( $P < 0.05$ ). Bars represent standard error.

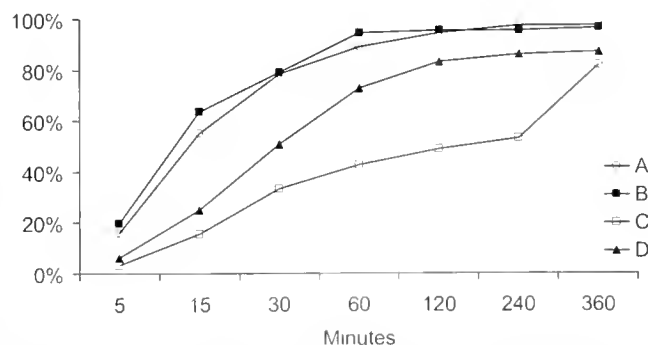


Figure 3. Time needed by *P. pansa* snails for the reattachment to the surface after removal. A = Aperture down on a wet surface, B = Aperture down in water, C = Snails on the back of the shell on a wet surface, D = Snails on the back of the shell in water.

alone be blamed for its declining population. The main reason is the time needed for reattachment to the surface, especially when the snails are placed on their backs and in dry locations. The attachment of the snails after replacing them on the surface takes too long and does not prevent them being washed away by the incoming tides. The period from "milking" until the attachment is a very critical phase for these animals. In the laboratory tests it took 2 hours for reattachment for 50%, and six for 80% of the animals placed on the back and on a wet surface. As soon as the animals are attached by their large foot muscle to the surface they are relatively safe from being displaced. Additionally the strong shell gives *P. pansa* protection against destruction by the waves. The removal of the purple snail from rocks causes a never-before-experienced situation and stress to the snails. Individuals respond in different ways by needing different periods for reattachment to the surface. The careful replacement of the snails after their removal from the rocks with the aperture down, on a moist surface or even in water, helps them to recover from the stress, and to reattach themselves. However, even under the most careful conditions of replacement some snails need long periods before reattachment and are in danger of being washed away during the approaching high tides with their high impact waves.

Today's commercial "ink-collectors" work under piecework conditions: the larger number of skeins dyed with the secretion of the snails in less time results in higher profits, but also inevitably in a larger number of snails "milked" and less care for their reattachment and conservation (Acevedo-García 1995). In the past a similar situation existed in Costa Rica where the indigenous people had to fulfill the demands for "hilo morado" by the Spanish ad-

ministrators and priests. This rush of work often results in the snails not being removed carefully from the rocks, not "milked" with care and at the end are left exposed to the strong wave actions of the sea or to the sun thus causing the death of the animals. Additionally, because female snails reach larger sizes than males and because of their larger size expel more secretion, they are preferred by "ink" collectors. Furthermore, the collection, especially of larger-sized purple snails as a special food for foreigners, is a recent development, which causes a reduction in recruitment and a decrease in the snail's population. However, not only the activities of collectors of the purple snail but also the increased effects in recent years of water contamination can be blamed for the decrease in snail populations (Castillo-Rodríguez & Amescua-Linares 1992).

A strict enforcement of the prohibition of collecting *P. pansa* is needed for the conservation of the purple snail, yes, even the prohibition to remove snails from the crevices of wave swept rocks. Additionally intensive research is required for the controlled reproduction to restock over-exploited areas.

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## ULTRASTRUCTURE OF SPERMATOGENESIS IN THE WHITE CLAM *CHIONE CALIFORNIENSIS* (BRODERIP, 1835) (MOLLUSCA: PELECYPODA)

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**ABSTRACT** Although studies of this species have focused on its reproductive cycle, there is no literature available on the ultrastructure of its germ cells. This study consequently aims to describe the latter cells as well as the shape of the acrosome, a character that may be of help in the taxonomic allocation of species of this family or other bivalve families. Results indicate spermatogonia have a central, spherical nucleus as well as scattered heterochromatin granules throughout the nucleoplasm. Rough endoplasmic reticulum is scarce, but the cytoplasm is rich in mitochondria and disperse glycogen granules. In primary spermatocytes, heterochromatin is more highly condensed and there is a reduction in the amount of cytoplasm and number of mitochondria as compared with spermatogonia. In secondary spermatocytes, heterochromatin is present in peripheral areas of the nucleus, forming occasional projections towards its center. In spermatids chromatin is fully condensed, taking up the entire nucleus. Mitochondria increase in size and migrate to the basal pole, giving rise, along with the centriole, to the spermatozoon neck. In spermatozoa, the nucleus is ovoid, the acrosome is round and the centriole is surrounded by four mitochondria, unlike other bivalves, like *Anadara tuberculosa*, in which the nucleus is reported to be round, the acrosome is pyramidal and there are five mitochondria.

**KEY WORDS:** white clam, *Chione*, spermatogenesis, ultrastructure

### INTRODUCTION

The white clam, *Chione californiensis* (Broderip, 1835) is a bivalve mollusc of the family Veneridae, a group regarded as a potential fishery and aquaculture resource (Baquero 1987). One of several species exploited by the traditional fishing industry along the coasts of the Sea of Cortés, it is for the most part a local food item (Baquero 1989). The reproductive cycle of several species of the genus *Chione* in Mexico has been described previously. García-Domínguez et al. (1993), in particular, have described the gonadal cycle of *Ch. californiensis*.

The ultrastructure of the spermatozoa has been described in many bivalve genera, such as *Bamia candida* (Pasteels & Harven 1962), *Hyriopsis schlegelii* (Higashi 1964), *Turtonia minuta* (Ockelmann 1964), *Mytilus edulis* and *M. perna* (Nijima & Dan 1965, Bourcart et al. 1965), *Spisula solidissima* (Longo & Anderson 1969), *Corbicula sandai* (Hachiri & Higashi 1970), *Crassostrea virginica* (Galtsoff & Philpott 1960, Daniels et al. 1973), *Bankia* spp. (Popham et al. 1974), *Lyrodus* spp. and *Teredo* spp. (Popham 1974), *Ligumia rostrata* (Trimble & Gaudin 1975), *Nucula hartvigiana* (Popham & Marshall 1977), *Anadara trapezia*, *Anomia descripta*, *Fulvia tenuicostata*, *Myodora brevis* and *Notocorbula vicaria* (Popham, 1979).

The ultrastructure of the different germ cells occurring during spermatogenesis and spermiogenesis was described in *Anadara granosa* by Suwanrajat and Parnrong (1999) and by Ortiz et al. (2003) in *A. tuberculosa*. Differences were found as to number of mitochondria and acrosome shape. Our study thus aims to describe the ultrastructure of these cells in *Ch. californiensis*, a factor that may help significantly to improve the taxonomic allocation of different bivalve species.

### MATERIALS AND METHODS

Male specimens were collected in an intertidal coral reef located north of Ensenada de la Paz, a coastal lagoon lying southwest of Bahía de la Paz, B.C.S., Mexico, at 24°06' to 24°10'N and 110°19' to 110°25'W. Small samples of gonad tissue were removed and fixed in 2.5% glutaraldehyde in seawater with a pH of 8 for 48 h, then rinsed with 2.5% sodium bicarbonate and postfixed with osmium tetroxide for 1 h (Buckland et al. 1986). Dehydration was done in three 10-min shifts with different concentrations of ethanol, followed by two 20-min propylene oxide shifts. Samples were then permeated with 1:1 propylene oxide/epoxy and embedded in Epon 812. Very thin 70-nm sections were obtained and set in mesh copper grids. These were treated with uranyl acetate and lead citrate to enhance contrast and later examined under the transmission electron microscope (Jeol-100SX).

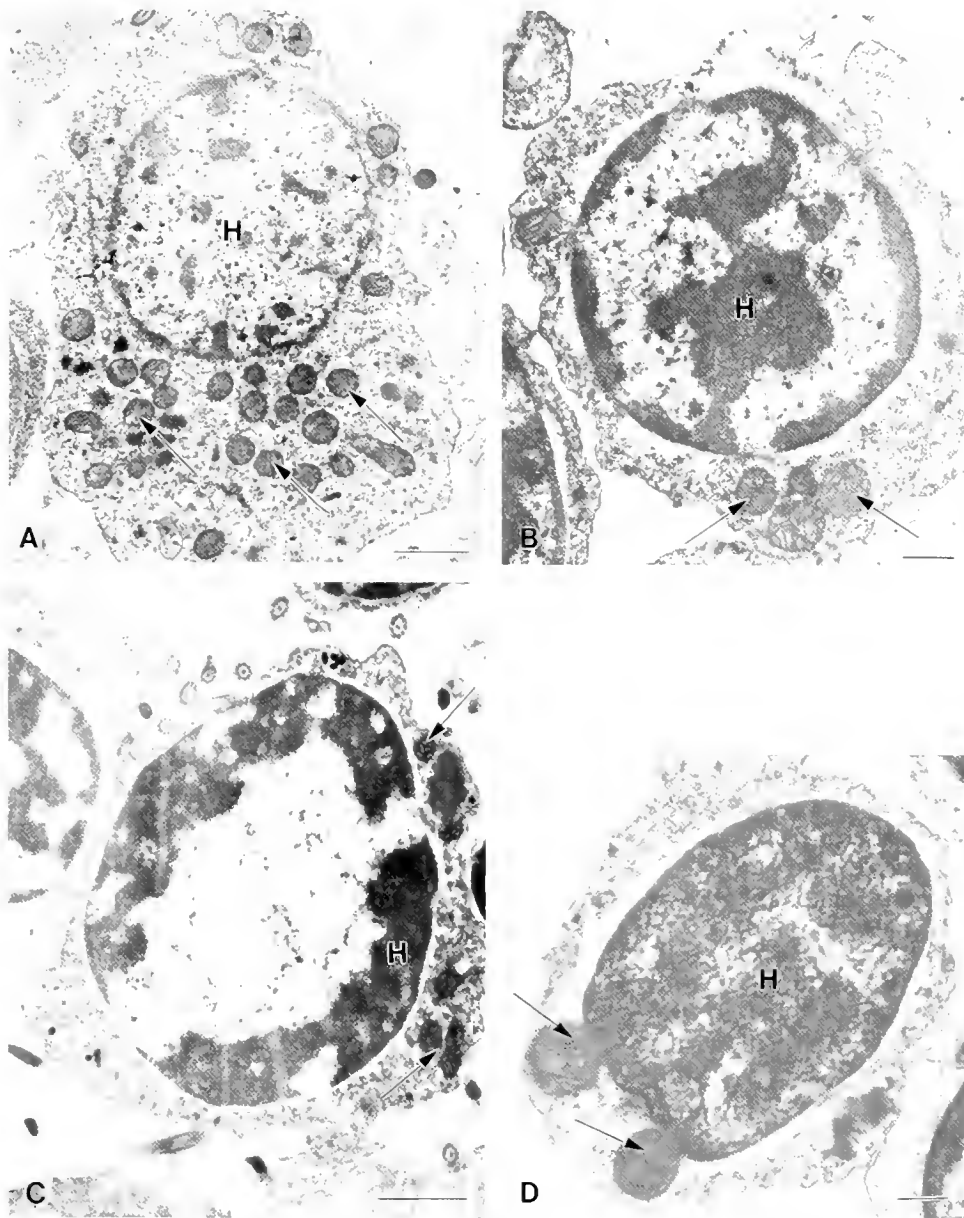
### RESULTS

The first germ cells to mature during spermatogenesis are spermatogonia, which have a large central nucleus and, typically, heterochromatin granules of varied size scattered throughout the nucleoplasm. The cytoplasm is rich in mitochondria, whereas rough endoplasmic reticulum, found near the nucleus, is scarce (Fig. 1A).

Primary spermatocytes are formed by mitosis of the spermatogonia. The nucleus has a larger amount of condensed heterochromatin, but there is less cytoplasm and fewer mitochondria than in spermatogonia (Fig. 1B). Primary spermatocytes give rise by meiosis to secondary spermatocytes. Heterochromatin is characteristically more condensed in the latter, migrating to peripheral areas of the nucleus (Fig. 1C).

Spermatids arise from secondary spermatocytes, which have become significantly smaller. There is less cytoplasm and it forms a halo around the nucleus. Chromatin takes up all of the latter and it is fully condensed. During this stage, mitochondria increase in size and migrate to the basal pole of the nucleus (Fig. 1D).

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**Figure 1.** (A) Spermatogonia. Randomly scattered heterochromatin (H). Abundant mitochondria (arrows) are shown. (B) Primary spermatocyte. Condensed heterochromatin (H) and scarce mitochondria (arrows) are observed. (C) Secondary spermatocyte. Heterochromatin is localized peripherally (H) and mitochondria (arrows) are shown. (D) Spermatid. Chromatin is completely condensed (H) and mitochondria are increased in size (arrows). Bar = 0.5  $\mu$ m.

Lastly, spermatids undergo a complex change during spermiogenesis giving rise to spermatozoa, which display three regions: head, middle piece and tail. The head is formed by an elongate nucleus and a round acrosoma (Fig. 2A). The neck or middle piece contains four mitochondria with well-developed cristae arrayed in a circle (Fig. 2B). Central to the four mitochondria lies the centriole, which gives rise to the flagellum. The latter exhibits the typical axoneme structure of nine paired peripheral microtubules and two central ones.

#### DISCUSSION

During the first two stages of spermatogenesis involving maturation of the spermatogonia, in the course of formation of the

primary spermatocytes, which undergo mitotic multiplication to give rise to secondary spermatocytes and during meiosis of the secondary spermatocytes in the course of which spermatids arise, germ cells contain a large central nucleus with heterochromatin granules of varied size and there are few organelles. In spermatids, chromatin is fully condensed and a reduced cytoplasm forms a halo about the nucleus, whereas mitochondria increase in size and migrate to the basal pole of the nucleus. These characters are similar to those reported by Suwanrajat and Pararong (1999) in *Anadara granosa* and Ortiz et al. (2003) in *A. tuberculosa*.

During spermiogenesis the name given to the last stage of spermatogenesis spermatids are fully transformed to give rise to spermatozoa. Existing characters confirm the primitive nature of the latter, as in several other bivalves with external fertilization (Franzén 1955).

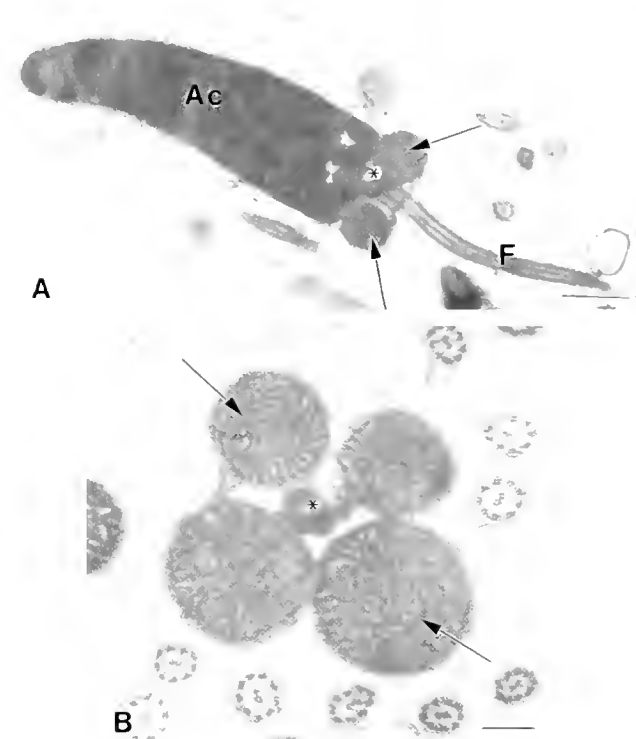


Figure 2. (A) Spermatozoid. Pyramidal acrosome (Ac), centromere (\*), mitochondria (arrows) and flagellum (F) are shown. (B) Typical four mitochondria (arrows) and centromere (\*). Bar = 0.5  $\mu$ m.

The acrosome of molluscs varies widely as to size, composition, morphology and position in spermatozoa. Arising usually in a vesicle derived from the Golgi body (Popham 1979), its function is to dissolve the various egg layers so that fusion of spermatozoon and ovum can occur (Colwin & Colwin 1967, Franklin 1970, Longo 1973). *Chione californiensis* has a round acrosome, unlike *A. tuberculosa* where the latter is pyramidal (Ortiz et al. 2003) and *A. granosa* in which it is triangular (Suwanrajat & Parnrong 1999).

The spermatozoon nucleus displays great morphological diversity in mollusc species. In spermatozoa of a primitive type the nucleus may be spherical, ovoid or conical (Popham 1979). Franzen (1955, 1970) mentions this structure is used as a systematic character.

Mitochondria are usually spherical in shape and their array as spherical masses on the proximal end of the spermatozoon head is also typical of species with external fertilization. Suwanrajat and Parnrong (1999) say the number of mitochondria may be species specific. Five mitochondria are present in *A. tuberculosa* (Ortiz et al. 2003) and four in *Ch. californiensis*, confirming the fact that this may be a species specific character.

Axoneme structure of the flagellum varies little in bivalve molluscs. It is comprised of a central pair of microtubules, which is surrounded by nine peripheral pairs (Ortiz et al. 2003). This concurs with our observations in *Ch. californiensis*.

We may conclude from this that the spermatozoa of *Chione californiensis* exhibit as species specific characters an ovoid nucleus, a round acrosome and a centriole surrounded by four mitochondria.

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## OUT-CROSSING AMONG COMMERCIAL STRAINS OF THE NORTHERN QUAHOG, *MERCENARIA MERCENARIA*: SURVIVAL, GROWTH AND IMPLICATIONS FOR SELECTIVE BREEDING

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**ABSTRACT** Because the accumulation of inbreeding within hatchery-propagated stocks of the hard clam, *Mercenaria mercenaria* (Linnaeus, 1758), could result in reduced growth and survival, we studied the potential for improving performance through out-crossing among existing hatchery strains. We produced all 10 possible out-crossed combinations among 5 strains of clams as well as all 5 pure parental strains simultaneously in the hatchery and measured their size at the time of metamorphosis (the spat stage) and at the end of a nursery period in mesh bags at a single field site (the seed stage). We then planted replicate plots of all fifteen strains at five field sites in Virginia, USA encompassing the range of salinity conditions used by commercial growers, and monitored growth and survival for two growing seasons. We found significant phenotypic differences among strains at the spat and seed stage, but different strains performed best at each stage. In the field we found significant site effects, strain effects and strain-by-site interactions, but there was no evidence of negative correlations in performance among sites indicating strong trade-offs that would be problematic for selective breeding. Three different linear contrasts designed to compare out-crossed and pure strains for each parental stock, test for nonadditive genetic effects within each pairing of different parental strains, and estimate the general combining ability of parental strains reveal a complex pattern. We found both inbreeding and out-breeding depression depending on the developmental stage of the clams and the parental strain examined. Within strain crosses generally produced larger spat but smaller seed. Out-crossed progeny were generally smaller at the spat stage than the average of their parental lines but larger at the seed stage. The two best performing parental strains had significant, positive, general combining abilities, whereas this measure was negative for the two worst parental strains. In the field, inbreeding depression was restricted to lines that showed poor pure strain performance, and these strains also showed poor general combining ability, whereas strains with good pure line performance showed out-breeding depression and good general combining ability. Only the poorest performing pure parental lines showed non-additive effects when we compared each out-crossed strain to the mean of its parental strains, suggesting that heterotic effects are unlikely to be useful for selective breeding. Finally, there were significant correlations between seed measurements and field performance indicating that it may be possible, in the context of selective breeding programs, to weed out inferior strains or families early in the life cycle.

**KEY WORDS:** *Mercenaria mercenaria*, hard clam, northern quahog, breeding, genetics, heterosis, inbreeding

### INTRODUCTION

On the eastern coast of the United States, the hard shell clam or Northern quahog, *Mercenaria mercenaria* (L.), is the most valuable aquaculture product produced. Wild populations historically supported a large subsistence, commercial and recreational fishery, but harvests have recently declined in many areas—probably caused by problems with water quality and overharvesting. On the other hand, the aquaculture sector of the hard clam market has been growing rapidly for the last 10–15 y and clams are now cultured from Massachusetts to Florida. USDA figures indicate that the economic value of the clam aquaculture market grew from approximately \$5 million in 1995 to approximately \$38 million in food products and \$1.2 million in seed clams in 1998 (Anonymous, 2000).

Despite the coast-wide economic importance of this species and the clam farming industry, there have been relatively few genetic studies of *M. mercenaria*, and selective breeding and domestication efforts have been largely limited to informal programs of mass selection conducted by commercial seed producers without rigorous genetic analysis (Gallivan & Allen 2000). The few genetic data available, along with anecdotal information from growers and hatchery operators, indicate that traits of economic interest such as growth and survival are heritable in hard clams

(recently reviewed by Hilbish 2001). Chanley (1960) reported that selection was able to improve growth in a population started from just three crosses. Hadley et al. (1991) reported a wide range of heritability estimates for growth (0–0.43), and Rawson and Hilbish (1990) reported a heritability of 0.37 for growth during the first 6 mo postspawning. Commonly cultured strains differ markedly in susceptibility to QPX disease, indicating a high degree of genetic control over this trait as well (Ford et al. 2002, Ragone Calvo & Bureson 2002, Ragone Calvo et al. 2003). In addition, some aspects of shell coloration appear to have a relatively simple genetic basis (Chanley 1960). All indications, therefore, are that domestication and selective breeding of hard clams could result in marked improvement of economically important characters.

Complicating matters for would-be shellfish breeders and commercial growers alike, however, is the finding that genotype by environment interactions for growth and survival are common in bivalves, including *M. mercenaria* (Hilbish et al. 1993, Newkirk 1978b, Rawson & Hilbish 1990, Rawson & Hilbish 1991). As a consequence, genotypes that perform well in one environment may perform poorly under different environmental conditions, and it is unclear whether “generalist” genotypes that perform well under a wide range of conditions, such as those found in Pacific oysters (Langdon et al. 2003), can be found or created in hard clams. In the extreme case, genotype by environment interactions can take the form of strong genetic trade-offs in performance among environments such that good performers in one environment are neces-

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sarily poor performers in others, and breeders may have no choice but to develop environment-specific selection lines.

In addition, common procedures in commercial hatcheries such as mass spawning with complete mixing of gametes for fertilization and/or mixing of groups produced through more controlled fertilization procedures combined with aggressive sieving and sorting during the early stages (i.e., larvae, spat and seed) are not conducive to either selective breeding or the conservation of genetic diversity (Dillon & Manzi 1993, Hadley 1993, Newkirk 1978a). These practices contribute to high variance in the contributions of individual parents to the breeding population through either differential fertilization success, larval mortality, or fecundity and can lead to the rapid accumulation of inbreeding, genetic drift, and low effective population size as has been demonstrated in Pacific oysters (Hedgecock & Sly 1990). Unfortunately, if the limited information available from other bivalve species can be extended to hard clams, even relatively low levels of inbreeding may result in substantial reductions in growth and survival (Evans et al. 2004). Commercial-scale hatcheries are typically equipped with a relatively small number of very large tanks for larval culture and thus are poorly equipped to prevent inbreeding beyond attempting to include many parents in the hopes that few are related, and balancing the genetic contributions of parents at the time of fertilization (M. Camara and S. Allen, pers. obs). Unfortunately, a recent study in Pacific oysters (Taris et al. 2006) found that in larval cultures consisting of a mixture of families, the relative contributions of the families could change rapidly, especially if the slow-growing larvae are culled through sieving. In addition, these strategies cannot ameliorate the effects of even infrequent population bottlenecks, and as a result, hatchery strains are likely to have lower genetic diversity and be more inbred than wild populations as has been found in unselected hatchery strains of the Suminoe oyster, *Crassostrea ariakensis* (Zhang et al. 2005) and strains of the Eastern oyster, *Crassostrea virginica* selected for disease resistance (Carlsson et al., in review, Hare et al. 2006). Thus, out-crossing among genetically distinct strains may lead to marked improvement in performance as has been demonstrated in other bivalve species (Hedgecock et al. 2004, Hedgecock et al. 1996).

As a first step toward designing and implementing a genetically rigorous domestication and selective breeding program for hard clams at the Aquaculture Genetics and Breeding Technology Center in Gloucester Point, Virginia, we evaluated the performance of five genetic strains that are used extensively by commercial clam growers in the midAtlantic region of the USA, both as within-strain crosses and in all possible pairwise combinations. Though we have no pedigree information from which to estimate the levels of relatedness among the parents used in any of our crosses, individuals from the same strain are much more likely to be related to each other than individuals from different strains. As a matter of convenience, therefore, we will sometimes refer to the within-cross strains as "pure strains" or "inbred strains" and the among-strain crosses as "hybrids" or "outbred" lines. We deployed all 15 genetic groups across a range of environments chosen to represent the spectrum of conditions under which hard clam aquaculture is practiced in Virginia. This experimental design allowed us to make comparisons of the performance of pure strains to each other, of each pure strain to outcrosses with the other four pure strains, of outcrossed strains derived from different combinations of parental strains and of outcrossed strains to predictions based on additive inheritance from their parental strains. In addition, we were able to

determine if these relationships were stable across varying environmental conditions. Finally, by collecting data on growth and survival at several stages in the life history, we could evaluate the strain-level correlations among characters and the stability of these correlations across environments, to address questions about whether performance in the field was predictable from data gathered in the hatchery or nursery phases of hard-clam culture.

## MATERIALS AND METHODS

### Broodstock Sources

Researchers in Virginia and New Jersey (Ford et al. 2002, Ragone Calvo & Bureson 2002, Ragone Calvo et al. 2003) have found marked differences in QPX resistance among strains produced in different regions when planted in common garden experiments. Therefore we selected a similar array of stocks for our study. However, because these studies found that the Florida stock they tested was highly susceptible to QPX, and because this finding resulted in a ban on the importation of clam seed from states south of Virginia into Virginia waters (<http://www.mrc.virginia.gov/regulations/fr754.shtml>), we did not use a Florida strain. Clam stocks were obtained from Massachusetts (MA), New Jersey (NJ), and South Carolina (SC), and two from Virginia (KK and VA). All strains except for VA were obtained from commercial hatcheries. The VA strain was the product of early, but limited, domestication efforts at the Virginia Institute of Marine Science (VIMS) led by Michael Castagna.

### Spawning, Larval Culture and Field Nursery

All parental animals were brought to the VIMS shellfish hatchery in Wachapreague, Virginia in March of 2000 and brought into reproductive condition by holding them in static tanks at 19°C to 23°C, and feeding them with high concentrations of a mixed diet of 3 species of cultured algae (*Isochrysis galbana*, *Tetraselmis chuii*, and *Chaetoceros neogracilis*). Water was changed three times per week. A few animals were checked weekly for gonadal development and gamete maturity by opening their shells and examining gametes microscopically. Fertilization is external in *M. mercenaria*, and females do not store sperm. Thus, to produce pairwise matings without contamination caused by having males and females in the same spawning raceway, we first determined the sex of individual animals by spawning all parents on 30 March 2001 using a combination fluctuating temperature (Hadley et al. 1997) and the addition of microwave-killed sperm to the spawning trays. We then marked each animal individually and recorded its gender. Males and females were subsequently reconditioned for spawning in separate tanks using the same methods as mentioned earlier. On May 17, 2001, we respawned males and females in separate raceways using the same techniques as mentioned earlier.

We first obtained gametes from each of the parents in separate containers. To accomplish this, at the first sign of spawning, individual parents were removed from the spawning raceway, rinsed several times with filtered seawater, and placed in individual beakers filled with filtered seawater where they continued to release gametes. Males that refused to release sperm were strip-spawned by opening their shells, lacerating the gonads with a scalpel, and rinsing sperm into beakers with filtered seawater. We then combined eggs and sperm to produce the desired crosses among individuals.

To ensure that all groups were as representative as possible of

their parent strains, and that the initial contributions of parents were reasonably balanced, we attempted to produce 20 full-sib families nested within each pure strain and hybrid cross at the time of fertilization and combined them one hour later. Gametes from as many as 10 males and 10 females from each parental strain were collected individually as described above. The eggs from each female were divided into six equal aliquots: one aliquot for each of the four hybrid groups involving that stock and two aliquots for within-strain crosses. Each aliquot was fertilized with sperm from a different male from the appropriate strain to produce full-sib families. For the hybrid groups the different males from each of the two parental strains were randomly paired with an equal number females from the other strain. The outbred strains were thus represented by up to 20 full-sib families derived from a total of up to 40 parents. Half of these families paired males from one parental strain with females from a second strain, and half paired females from the first strain with males from the second. For the five pure strains, the crosses were slightly different. Because the number of parents within each strain was limited, we used each male and female as parents for two different full-sib families. That is, sperm from each of the males was used to fertilize aliquots of eggs from two different females. As a result, the up to 20 full-sib families within the five pure strain crosses are actually a mixture of full and half-sib families derived from a total of up to 20 parents, each of which contributed to two single-pair crosses. After fertilization was complete, all of the families, representing each pure or hybrid strain, were pooled to form 15 groups representing all possible combinations of the five parental strains. For two of our parental strains, we were unable to spawn 20 parents. Specifically we used only eight males and eight females from the SC stock, and only five males and five females from the VA stock.

Each of the resulting 15 pooled cultures was then split into two 200 L static larval rearing tanks, each containing approximately 1.5 million fertilized eggs to ensure against the loss of cultures and to distribute each group across potential environmental gradients in the hatchery. Tanks were maintained at 25°C and the developing larvae were fed cultured *Isochrysis galbana* twice daily until metamorphosis.

After setting, seed from the two larval tanks for each cross were pooled, and we reared the juveniles first in downwellers and then in upwellers in an outdoor nursery system supplied with unfiltered seawater from the estuary at Wachapreague, VA. On July 20, 2001, when the seed clams had reached about 3–5 mm in shell length, they were transferred to fine mesh spat bags and deployed on the bottom of Cherrystone Creek, VA until October 10, 2001 when they had reached about 10 mm in shell length. At this point, the spat bags were retrieved and the animals from each group were divided into 15 aliquots of 1,250 animals each as estimated volumetrically based on group-specific determinations of volume/count relationships. Poor survival to this point of the VA × VA line permitted only 9 aliquots.

#### Field Sites and Grow-out

We then deployed the animals at 5 field sites chosen to represent the range of conditions used by commercial clam growers in Virginia. Site locations are shown in Figure 1 and include two high salinity "sea-side" locations in estuaries on the eastern side of the Delmarva peninsula (Quinby and Wachapreague Inlet), two mid-salinity sites in the lower Chesapeake Bay (Hungar's Creek and York River), and one low salinity site (North River). The two

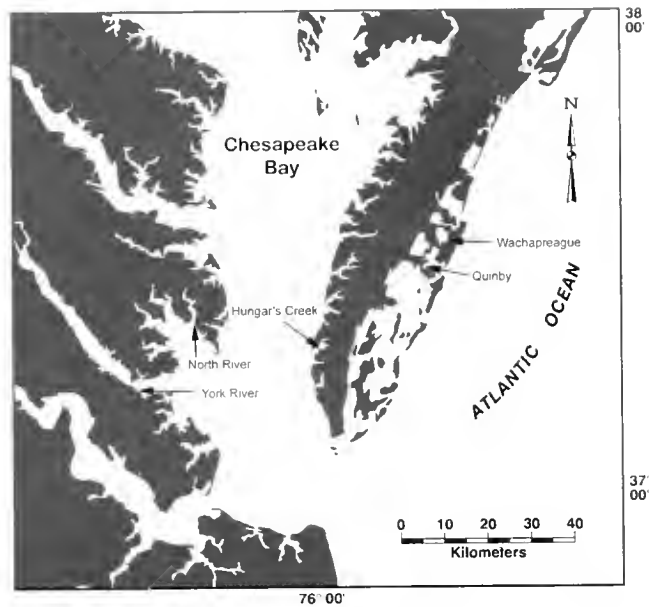


Figure 1. Map showing the locations of the five field sites in the Virginia portion of the Chesapeake Bay.

seaside sites experience high and stable salinity generally between 30‰ and 35‰. Salinity at the York River and Hungar's Creek sites varies seasonally from a low of about 12‰ in spring to nearly 25‰ in fall/winter. ([http://www.vims.edu/data\\_archive/pier/figs/others.html#sal](http://www.vims.edu/data_archive/pier/figs/others.html#sal)). The North River site is even more variable. Salinities at this site be as low as 0‰ after severe rain events and as high as 25‰ during extended droughts (K. Kurkowski, pers. comm.) At each site, we planted three replicate 1.52 m × 1.52 m plots, each seeded with 1,250 animals that had been previously aliquoted with the exception of the pure VA line, which was not planted at the Hungar's Creek and North River sites. The Wachapreague site was planted on October 11, Quinby on October 12, North River on October 15 and Hungar's Creek and York River on October 16. The 45 plots at each site were arranged in a rectangular grid in a completely randomized design with plots separated by 0.91 m. Each plot was covered with 6.5 mm plastic mesh to exclude predators, and the mesh was held in place by lengths of steel concrete reinforcement bar pressed into the substrate at the edges of the plots as is typical for commercial clam culture in Virginia. Plots were visited regularly to clean and maintain the predator exclusion nets, especially after damaging winter storms.

#### Sample and Data Collection

Samples of spat and seed were collected and preserved in ethanol at 2 stages in the process: (1) at the end of the nursery stage when the animals were transferred to fine mesh bags in the field, hereafter referred to as "spat" and (2) at the time of field planting, hereafter referred to as "seed." We measured the size of spat using image analysis. Approximately 100 randomly selected spat from each group were spread so that no two were in contact with each other in a clear plastic 150 mm diameter Petri dish placed on a light table and photographed using a digital camera mounted in a copy stand. To minimize parallax error, we positioned the camera approximately 60 cm above the dish and used the camera's zoom lens feature to fill the frame rather than placing the camera close

to the dish. A ruler was included in the frame for calibration. Because of their bilaterally symmetric shape, all spat naturally assume the same position with one valve on the surface of the dish and the other facing the camera. We then used SigmaScan Pro image analysis software (SPSS Inc.) to quantify the area presented to the camera in  $\text{mm}^2$  by each of the spat in the digital image. For seed, we measured the shell length and shell width of a random sample of 20 animals from each group to the nearest 0.01 mm using digital calipers. Shell length was defined in this study as the longest dimension of the whole animal in anterior-posterior direction. Shell width was the longest dimension of the whole animal in the lateral dimension. We did not measure shell height (the longest dimension in the dorsal-ventral dimension).

Once the animals were planted in the field, we collected samples from the plots in the Autumn (Oct/Nov) of each year of the study (2002 and 2003). At each sampling date, we removed the net from each plot and removed 4 randomly located 10.2 cm diameter  $\times$  25 cm deep cores of sediment and all of the animals they contained. We sieved each of the cores in the field through 6.5 mm mesh and all live animals and empty shells were collected and counted to estimate the number of live clams in the plot. If we collected fewer than 20 animals in the core samples from any plot, we randomly collected more animals from that plot until we had collected 20 live animals for size measurements. We then brought all the live animals and shells back to the laboratory and measured their shell length and shell width to the nearest 0.01 mm using digital calipers.

#### Statistical Analyses

All statistical analyses were conducted using SAS version 8 (SAS, 2000). We first examined the frequency distributions and normal probability plots for all of our measurement data and found no marked departures from normality. We, therefore tested for overall differences among the genetic groups in the size of spat and seed using 1-way analysis of variance (ANOVA) with group as the single fixed factor with 15 levels and spat area, seed shell length and seed shell width as response variables. In these analyses, we used the among-individuals residual mean square as the error term in hypothesis tests because mixing the two larval cultures at metamorphosis made them effectively unreplicated.

We followed these ANOVAs with three sets of linear contrasts. The first set of contrasts compared the mean of each of the five pure strains to the pooled mean of the other four outbred strains in which it was represented as a parent. We refer to these as "pure versus hybrid" contrasts. The second set of contrasts ignored the pure lines altogether and for each of the five parental strains, compared the mean of the four hybrid strains to which it contributed to the mean of all 10 outbred lines. These contrasts estimate the average impact of each parental stock on the performance of hybrid progeny, typically referred to as "general combining ability" in analyses of within-population genetic variation (Falconer & Mackay 1996). We refer to these contrasts as general combining ability contrasts even though our experiment involves crosses within and among outbred stocks rather than the more typical inbred lines or individual genotypes. The third set of contrasts tests for nonadditive contributions of the pure parental strains (i.e., dominance and epistasis [Falconer & Mackay 1996]) in outcrossed hybrids by comparing each of the 10 outbred strains to the mean of the two inbred strains from which it was created. For brevity, we refer to these as "mid-parent" contrasts even though we did not

collect any data on the actual parents but rather on the pure parental strains reared simultaneously.

Unlike the larval and nursery stages, each strain was planted in replicated plots at all sites in the field (with the previously noted exception that the VA pure line was not planted at two of the five sites). We therefore tested for differences among-sites and groups in survival (quantified as the number of live animals collected in the core samples) and both of the size-at-age measurements using 2-way ANOVA. The linear model included fixed effects of site and group, site-by-group interaction and, except for survival (which can only be measured on entire plots) a random effect of plot nested within site/group combinations. We also tested for differences between pure and hybrid strains within each of the sites using the same three sets of contrasts as earlier mentioned following separate 1-way analyses of variance for each site with group as the only factor.

To address whether significant site-by-group interactions take the form of strong trade-offs such that groups that perform well in one site tended to perform poorly in other sites, we tested all possible pair-wise correlations between the group means among sites with the expectation that trade-offs would manifest themselves as significant negative correlations.

Finally, because we wanted to determine if performance in the field was predictable from data collected in the hatchery or spat bag stage of culture, we tested for correlations between the group means for spat area and seed length at planting and the group means for survival and shell size measurements at each of the field sites.

## RESULTS

#### Hatchery/Nursery Characters: Spat and Seed Size

There were significant overall differences among the 15 genetic groups in both the mean size of spat as measured by their areas and the mean size of seed as measured by shell length and shell width (Table 1a). Looking first at the spat area data, an examination of Figure 2a reveals that immediately post metamorphosis, the pure NJ  $\times$  NJ line produced much larger spat than any of the other groups, that the KK  $\times$  KK and MA  $\times$  MA pure lines are larger (though less dramatically) than the others, and that all of the outcrossed lines that have the KK strain as one parent tend to be slightly larger than the remaining groups. The pure versus hybrid contrasts (Table 1b) show significant positive effects on spat area of crossing within strains (or conversely negative effects of outcrossing) for the KK, MA and NJ lines, no effect for the SC line, and a significant negative effect of inbreeding for the VA line. The general combining ability contrasts (Table 1c) show that the KK-containing hybrid lines produce significantly larger spat than the average outcrossed strain, consistent with the observation earlier that lines with a KK parent are larger at the spat stage than others (Fig. 2a). In contrast, out-breeding the MA and VA lines to produce hybrids resulted in significantly smaller spat than the average outbred group as indicated by significant negative parameter estimates in the general combining ability contrasts, and hybrid lines that include the NJ or SC line as one parent do not differ from the mean of all outbred groups at the spat stage (Table 1c).

Because seed shell length and seed shell height were highly correlated ( $r = 0.995$ , data not shown), and produced essentially identical statistical results, we present results for seed shell length only. There are significant differences among the 15 genetic groups in an overall analysis (Table 1a), but these effects are less

TABLE 1.

Analysis of variance results for spat and seed measurements. A. Overall tests of heterogeneity of means of the 15 genetic groups produced. B. Parameter estimate and significance tests of linear contrasts comparing the mean of each pure strain to the mean of the four hybrid strains to which it contributed. Positive parameter estimates indicate that pure lines were superior to hybrid lines. C. Parameter estimates and significance tests of linear contrasts comparing the mean of the four hybrid groups that have the focal group as one parent to the mean of all 10 hybrid groups produced. Positive parameter estimates indicate that the focal line for a given contrast tended to produce superior progeny. D. Parameter estimates and significance tests of linear contrasts comparing each hybrid group to the mean of its two pure strain parental groups. Positive parameter estimates indicate that hybrid strains performed better than the average of the two parental stocks.

## A. Overall ANOVA results

Group	Spat area		Seed length	
	F	P	F	P
	69.75	***	24.54	***

## B. Pure vs. hybrid contrasts

Parent strain	Parameter	P	Parameter	P
KK	1.39	***	-0.95	**
MA	2.62	***	-1.54	***
NJ	6.38	***	0.06	ns
SC	0.19	ns	-0.72	*
VA	-1.97	***	-2.95	***

## C. General combining ability contrasts

Parent strain	Parameter	P	Parameter	P
KK	0.89	***	0.32	**
MA	-0.43	***	-0.46	***
NJ	0.07	ns	0.16	ns
SC	-0.02	ns	0.60	***
VA	-1.97	***	-0.62	***

## D. Mid-parent vs. out-crossed contrasts

Hybrid	Parameter	P	Parameter	P
KK × MA	-3.55	***	2.51	***
KK × NJ	-5.62	***	1.08	ns
KK × SC	-0.31	ns	1.80	**
KK × VA	1.22	ns	5.18	***
MA × NJ	-8.21	***	0.64	ns
MA × SC	-3.87	***	4.16	***
MA × VA	-3.02	***	1.10	ns
NJ × SC	-7.61	***	2.37	***
NJ × VA	-5.95	***	2.63	***
SC × VA	2.51	***	2.92	***

ns = not significant, \* =  $P < 0.05$ ; \*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$ .

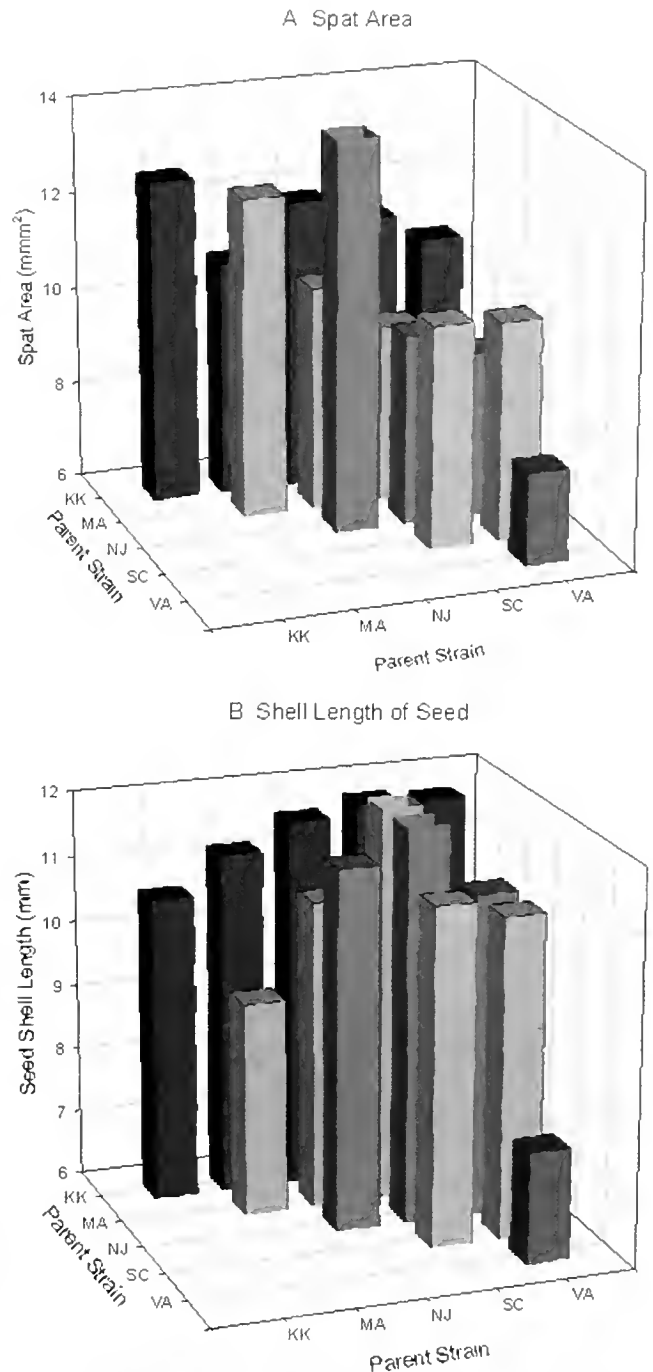


Figure 2. Size measurements for spat and seed. A. Spat area for all 15 possible combinations of the 5 parental stocks. B. Seed shell length.

attributable to the differences seen for the NJ, KK and MA pure lines spat area data. Rather, the most obvious patterns are that all groups that have either a KK or SC parent tend to produce larger seed than those that do not (Fig. 2b). The general combining ability contrasts among outbred strains (Table 1c) confirm this. Seed from KK- and SC-containing hybrid lines were significantly larger than the average outbred strain, and MA- and VA-containing outbred lines were significantly smaller. Contrasts comparing the pure versus hybrid progeny of each parental strain (Table 1b) show significant inbreeding depression of seed shell length in the KK, MA,

VA and SC lines, but these effects are much stronger in the VA and MA lines than in the K and SC strains. There were no significant effects of inbreeding on seed size in the NJ line.

The midparent contrasts (Table 1d) for spat area show significant negative effects for 7 of the 10 hybrid lines indicating that out-crossed spat are typically smaller than the average of their parental stocks. There was also one significant positive effect and two of these contrasts were nonsignificant. For seed shell length, seven of the possible contrasts are significant. All of these significant tests have positive parameter estimates indicating that at the

TABLE 2.

Overall analysis of variance results for field measurements in Autumn 2002 and Autumn 2003 testing for heterogeneity of means of the 15 genetic groups produced and group-by-site interactions.

	Autumn 2002				Autumn 2003			
	# Live		Length		# Live		Length	
	F	P	F	P	F	P	F	P
Site	35.18	***	142.04	***	3.97	*	63.01	***
Group	3.59	***	32.49	***	1.94	*	22.62	***
Site * Group	0.86	ns	1.44	*	1.01	ns	1.4	ns
Plot (Site * Group)	—	—	3.74	***	—	—	3.65	***

ns = not significant, \* =  $P < 0.05$ , \*\* =  $P < 0.01$ , \*\*\* =  $P < 0.001$ , — = not testable.

seed stage, most hybrid crosses are larger than expected by additive contributions of the two parental strains.

#### Field Characters: Size-at-Age and Survival

The overall analyses of variance for the survival and size-at-age measurements (Table 2) reveal that mean survival and shell length in the field differed significantly among field sites and among genetic groups at the Autumn 2002 and Autumn 2003 samplings.

For shell length in Autumn 2002 there was also a significant interaction effect between site and group but not for survival in either year or shell length in 2003. An examination of the pattern of survival in the field among sites reveals that the Site effects on survival in 2002 (Fig. 3) are largely caused by much higher survival at the Wachapreague site than all other sites and lower survival of all groups at the Hungar's Creek and Quinby sites, whereas by 2003 the number of survivors is highest at the Quinby site (Fig. 4). It should be noted, however, that these data have some limitations. Our cores samples, for example, captured more live animals in 2003 than in 2002 at the Quinby site. One possible explanation is that the soft, muddy bottom at the Quinby site caused us to under sample these plots in 2002 because the clams were deeper than our core samples. This is, however, very unlikely to have been a problem at other sites where the bottom was firm sand.

For shell length in the field (Fig. 5 and Fig. 6), the significant main effect of sites is largely driven by the larger size of animals at the York River site and smaller size in Hungar's Creek in 2002. In 2003, site effects are less dramatic, partly owing to the loss of the York River site to Hurricane Isabel, but animals at the Quinby, Wachapreague and Hungar's Creek sites are clearly larger overall than at North River.

Pure versus hybrid contrasts for each parental strain at each of the 5 sites (Table 3a) reveal mixed effects. The KK strain showed

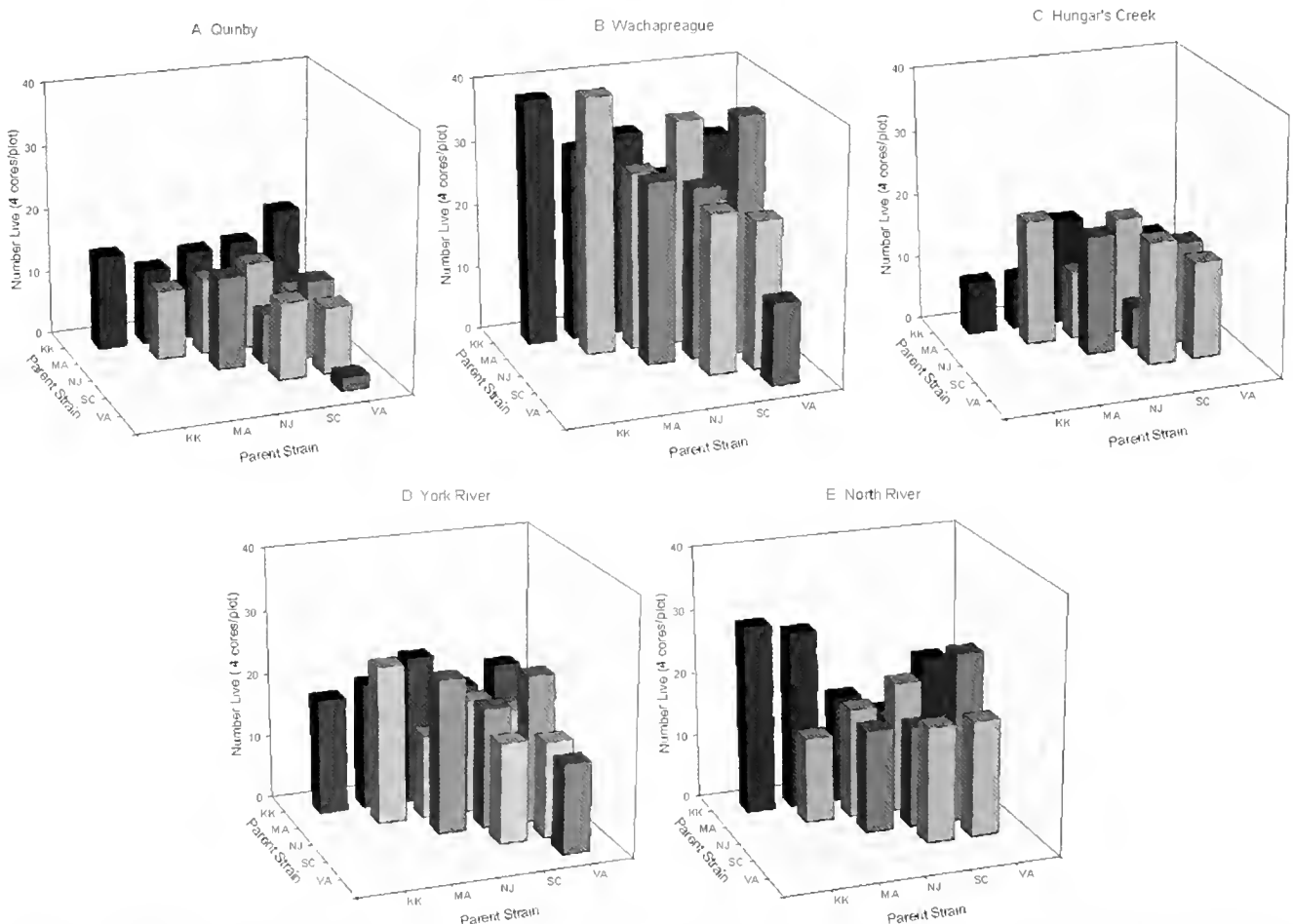


Figure 3. Survival at the Fall 2002 sampling date of all possible combinations of the 5 parental stocks at each of the 5 field sites as represented by the number of live animals collected in the four core samples.

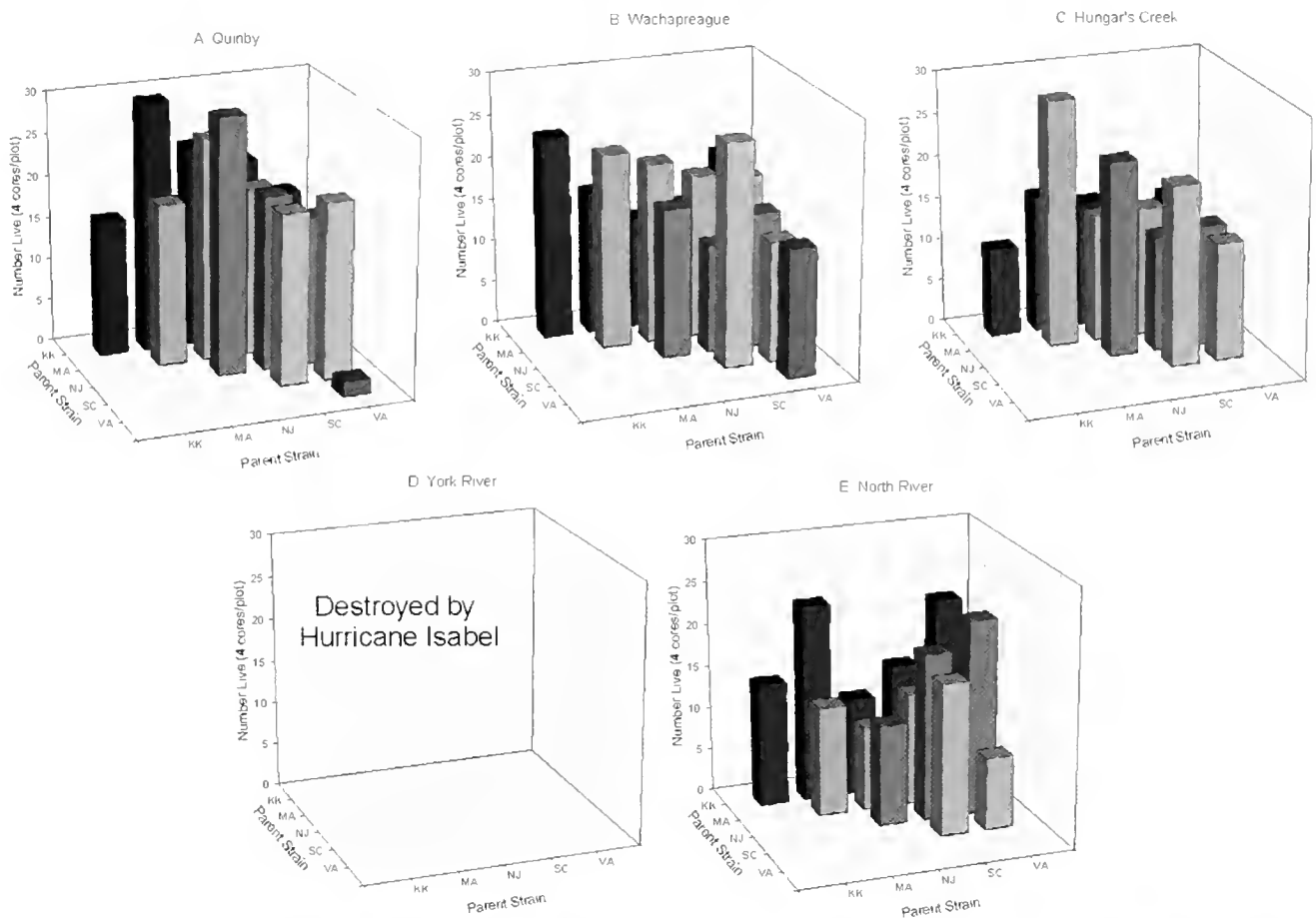


Figure 4. Survival at the Fall 2003 sampling date of all possible combinations of the 5 parental stocks at each of the 5 field sites as represented by the number of live animals collected in the four core samples collected.

no significant comparisons. In contrast, the MA line shows a wide array of effects caused by crossing within the strain. When crossed with itself, the MA line shows higher survival at the York River site and reduced shell length at all sites except for Hungar's Creek in 2002. In 2003, pure strains of the MA line produced significantly higher survival at the Hungar's Creek site accompanied by reduced shell length at all sites. The NJ line shows no significant effects of inbreeding on any of our measurements at any of the sites in either year. Pure SC stocks were significantly larger in 2003 at the North River site, but there were no other differences. The VA line showed the most dramatic effects of crossing within-strain, with negative impacts at all sites where it was planted in both years except for survival in 2002 at the York River site and in 2003 at Wachapreague. All in all, the 2002 field data produced 10 significant pure versus hybrid contrasts out of a total of 46 possible tests, and of these 10, only 1 is positive indicating better survival of the MA pure strain relative to its hybrids lines at the York River site and 9 are negative indicating inferior growth or survival in the pure MA and VA lines at multiple sites. In 2003, however, of the 36 possible comparisons (owing to the destruction of the York River site), 2 show significant positive effects of within-line crossing (for survival of the MA line at the Hungar's Creek site and shell length of the SC line at the North River), and 7 are negative. Of these 7 all are in the MA and VA lines and 6 indicate inferior growth of pure crosses.

Comparing general combining abilities reveals strong differ-

ences in the pattern of significance among the parental lines (Table 3b). Groups with one KK parent are generally not significantly different from the average outbred strain at any site except for enhanced survival at the Quinby site in 2002 (Note, however, that this is the site where survival estimates are most suspect because of possible limitations of our sampling technique in the muddy substrate). Stocks with one MA parent survive in numbers equal to the average hybrid strain in both years, but are smaller at all sites in 2002 and at the North River site only in 2003. The NJ stock shows no significant general combining ability for any of the traits we measured. The SC parental stock shows no general combining ability for survival, but outbred strains with one SC parent are larger at all sites in both years. Stocks containing the VA parental line had lower than average survival until 2003 at the Quinby site and were smaller than the average outbred strain in both years with the exception of the Wachapreague site in 2003.

The midparent contrasts, evaluated separately at each site also show mixed effects (Table 3c). For survival until Autumn 2002, only 4 of the possible 42 contrasts are significant, with 2 being negative and 2 positive. In the autumn of 2003, of the 28 estimable contrasts only 1 shows a significantly positive nonadditive effect on survival. Many more of these contrasts are significant for shell length, with 15 of 42 significant in 2002, and 5 significant in 2003. In 2002 and 2003, all of the significant contrasts for shell length are positive, indicating that when these contrasts are significant, hybrid progeny are larger than expected from additive contribu-



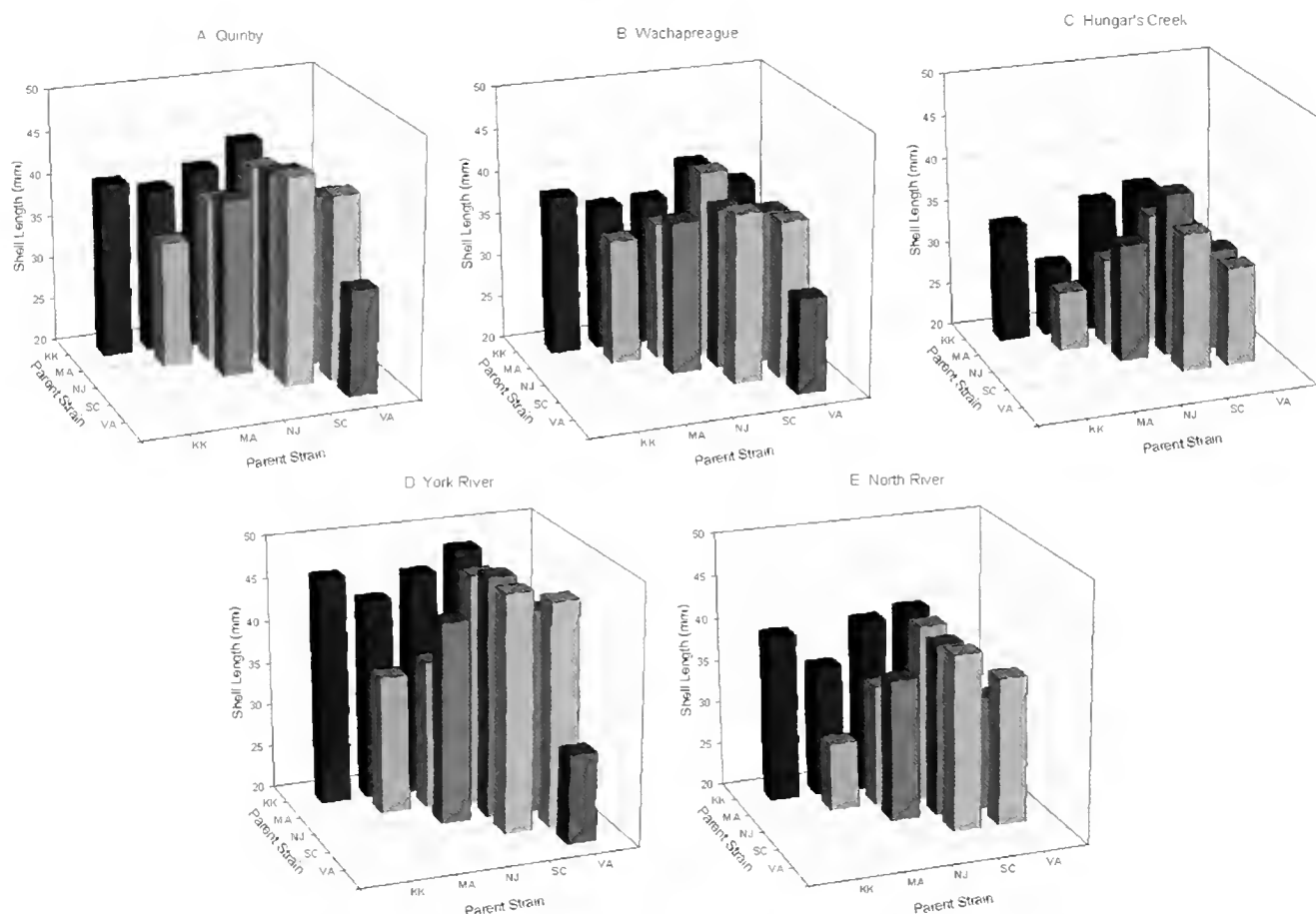


Figure 5. Shell length (largest dorso-ventral dimension) at the Fall 2002 sampling date of all possible combinations of the 5 parental stocks at each of the 5 field sites.

tions of parental strains. Further nearly all of the significant contrasts involve either the VA or MA parental lines as parents with the only exceptions being a significant positive effect on length for the NJ  $\times$  SC cross at the North River site in both years.

Table 4 shows the group-level among-site correlations for survival and shell length in both Autumn 2002 and Autumn 2003. The survival data (Table 4a) produced only one statistically significant correlation, a positive relationship between survival at the Wachapreague and York River sites in 2002 (note, however, that the York River site was destroyed before the 2003 sampling period). Shell length (Table 4b) is positively correlated among sites for all pairwise combinations of field sites in both years, with correlation coefficients ranging from 0.7–0.95.

#### Correlations Between Hatchery/Nursery and Field Measurements

Table 5 contains the correlation matrices among the group means of the two adult characters we measured and between juvenile and adult characters. The hatchery traits (spat area and seed length) are significantly correlated with each other ( $r = 0.311$ ). The correlation between adult shell length and the number of survivors is significant in 2003 ( $r = 0.223$ ), and at the Quinby site in both 2002 ( $r = 0.525$ ) and 2003 ( $r = 0.557$ ). There are, however, significant correlations between seed length, but not spat area, and both adult dimensions with correlation coefficients ranging from about 0.6 to nearly 0.9.

#### DISCUSSION

This study reveals a number of patterns that should be taken into account by both commercial clam growers and by breeders seeking to develop domesticated and genetically improved hard clam strains. In the field, it is no surprise that site selection has strong influences on the average survival of the genetic strains we produced in this study (Fig. 3 and Fig. 4) and weaker effects on growth (Fig. 5 and Fig. 6). Both clam culturists and breeders should place a high priority on survival, with growers seeking sites that maximize survival and breeders seeking to improve and to stabilize survival across a range of conditions. This is consistent with the finding that in Pacific oysters survival is more genetically variable than growth (Ernande et al. 2004). Less pronounced, but also important are the consistently higher growth of specific strains regardless of the site at which they are planted. Despite statistically significant group by site interactions in the overall analyses, all of the groups that had one parent from either the KK or SC parental strains tended to be larger at all of our test sites, and there were no negative among-site correlations for survival or size-at-age measurements (Table 4). The implications of this for breeders are that if our results can be generalized beyond the small number of strains tested here, it may not be necessary to develop specialty strains for different environments because the same strains performed best in all environments despite significant genotype-by-environment interactions.



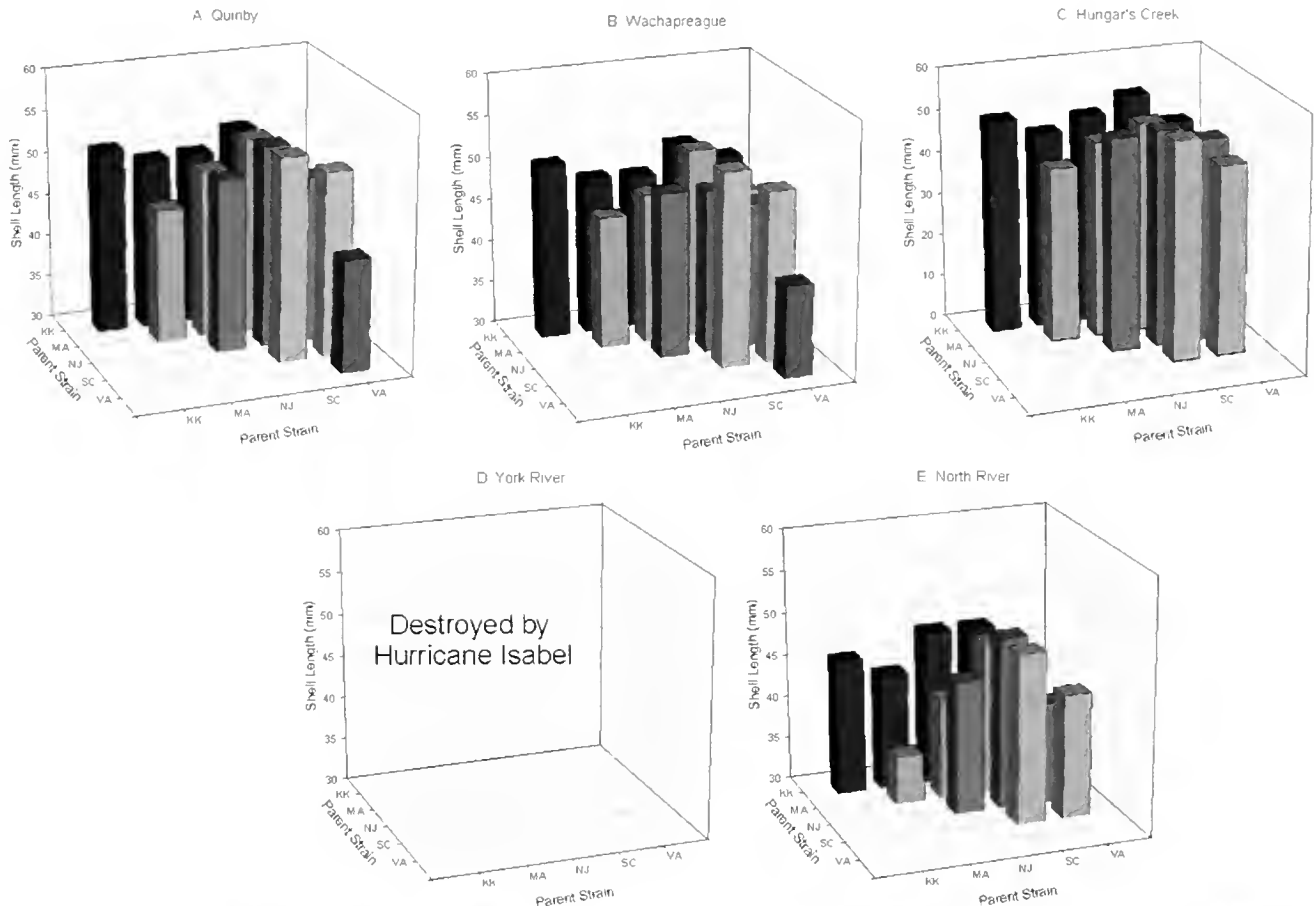


Figure 6. Shell length (largest dorso-ventral dimension) at the Fall 2003 sampling date of all possible combinations of the 5 parental stocks at each of the 5 field sites.

While we cannot be certain of the history of the stocks we used, all have been subjected to informal selection for a number of generations, and in some cases a combination of informal selection and inbreeding may have purged a substantial proportion of the deleterious alleles resulting in superior performing strains that can tolerate inbreeding. The implication here is that some low level of inbreeding may not be as detrimental as expected if it is accompanied by selection against deleterious alleles. The interplay between inbreeding and selection can be complex, especially in the presence of epistasis, genotype  $\times$  environment interaction, or genetic correlations. While there is still a great deal of both theoretical and empirical work required in this area (see Chap. 10 in Lynch & Walsh 2000), our data suggest that more precise studies of inbreeding and selection could be useful in designing an effective selective breeding program for hard clams.

In addition, breeders and broodstock managers must take into account their specific goals when making selection and management decisions. If, for example, the goal of a selective breeding program is to develop high-performance strains for use in aquaculture, our data indicate that some balanced between inbreeding and selection may purge deleterious alleles and produce high performing lines with little or no inbreeding depression. On the other hand, if the goal is to produce a genetically variable stock (e.g., for ecological restoration) then purging alleles that are deleterious under current conditions could have the undesirable effect of constraining the ability of restored populations to respond to changing conditions or novel pathogens.

Inbreeding effects also vary in the course of development. Crossing within strains, evaluated as the comparisons between pure and hybrid strains (Table 1b) generally has positive effects on spat area relative to among-strain crosses, with the exception of the VA stock, but negative effects later in development as measured by seed length. The same pattern is clear in the comparisons of hybrid lines to their midparent values (Table 1d), with hybrid progeny consistently smaller than the mean of the two parental lines as spat, but larger as seed. The most likely explanation for this pattern is that selection has been more intense and consistent for improved larval and nursery performance than for growth in the field under commercial conditions. All clam hatcheries experience high levels of larval mortality during the larval phase and aggressively sieve their larval cultures to eliminate dead and slow-growing larvae. In addition, the conditions in different hatcheries are likely to be quite similar because density, temperature, and food availability are relatively simple to control. In the field, however, mortality is typically lower and conditions are more variable from site to site. As a consequence, selection is likely to have been less intense and/or more variable in the field. Thus, selection in hatcheries is more likely to fix either favorable recessive genes or beneficial epistatic gene combinations and out-crossing is more likely to have detrimental impacts on hatchery performance.

In the field, the effects of crossing within parental strains are more complex. The significant comparisons between pure and hybrid groups that indicate negative effects that may be caused by inbreeding depression were concentrated in just two of the five

TABLE 3.

Parameter estimates and significance tests of linear contrasts: A. Contrasts of the mean of each "pure strain" to the mean of the four hybrid strains to which it contributed. B. Contrasts of the mean of the four "hybrid" groups that have the focal group as one parent to the mean of all ten hybrid groups. C. Contrasts between each outcrossed hybrid group and the mean of its two pure strain parental groups.

A. Linear contrasts of pure vs. hybrid groups for each parent strain by site									
		Autumn 2002				Autumn 2003			
		# Live		Length		# Live		Length	
		Parameter	P	Parameter	P	Parameter	P	Parameter	P
Parent Strain	Site								
KK	Hungar's Creek	-3.67	ns	0.79	ns	-3.25	ns	1.79	ns
	North River	9.50	ns	2.34	ns	-3.41	ns	0.50	ns
	Quinby	0.08	ns	-0.34	ns	-6.83	ns	1.23	ns
	Wachapreague	9.33	ns	0.44	ns	7.88	ns	1.28	ns
	York River	-2.17	ns	1.69	ns	—	—	—	—
MA	Hungar's Creek	8.75	ns	-3.01	ns	15.50	*	-5.64	*
	North River	-4.50	ns	-7.02	***	-0.41	ns	-7.55	***
	Quinby	-0.05	ns	-4.79	***	-2.33	ns	-4.17	***
	Wachapreague	11.33	ns	-2.50	*	3.91	ns	-3.23	*
	York River	8.25	*	-5.79	*	—	—	—	—
NJ	Hungar's Creek	6.17	ns	-0.47	ns	8.50	ns	1.15	ns
	North River	-2.91	ns	-0.55	ns	-4.08	ns	0.10	ns
	Quinby	3.08	ns	-0.13	ns	8.17	ns	-0.42	ns
	Wachapreague	-2.41	ns	0.42	ns	1.79	ns	1.79	ns
	York River	4.58	ns	-0.20	ns	—	—	—	—
SC	Hungar's Creek	6.67	ns	0.63	ns	8.17	ns	2.00	ns
	North River	-1.08	ns	1.30	ns	3.83	ns	2.23	*
	Quinby	0.58	ns	1.66	ns	-0.50	ns	1.25	ns
	Wachapreague	-2.25	ns	-0.04	ns	11.13	ns	2.29	ns
	York River	-1.58	ns	0.39	ns	—	—	—	—
VA	Hungar's Creek	—	—	—	—	—	—	—	—
	North River	—	—	—	—	—	—	—	—
	Quinby	-10.25	**	-7.40	***	-14.67	**	-4.72	*
	Wachapreague	-15.33	*	-5.97	***	-1.88	ns	-7.42	**
	York River	-4.41	ns	-12.58	***	—	—	—	—

B. Linear contrasts for general combining ability of each parent strain by site									
		Autumn 2002				Autumn 2003			
		# Live		Length		# Live		Length	
		Parameter	P	Parameter	P	Parameter	P	Parameter	P
Parent Strain	Site								
KK	Hungar's Creek	-0.13	ns	0.21	ns	0.38	ns	-0.73	ns
	North River	1.53	ns	0.84	ns	2.68	ns	0.07	ns
	Quinby	2.45	*	0.19	ns	2.27	ns	-0.18	ns
	Wachapreague	0.27	ns	0.23	ns	-0.06	ns	0.36	ns
	York River	1.83	ns	0.81	ns	—	—	—	—
MA	Hungar's Creek	-1.21	ns	-2.53	**	-0.03	ns	-1.16	ns
	North River	-0.47	ns	-1.41	*	-1.98	ns	-1.52	**
	Quinby	-0.97	ns	-1.18	***	0.77	ns	-0.68	ns
	Wachapreague	-0.07	ns	-0.81	*	2.38	ns	-0.26	ns
	York River	-1.91	ns	-2.29	***	—	—	—	—
NJ	Hungar's Creek	0.37	ns	1.42	ns	0.97	ns	1.04	ns
	North River	-0.62	ns	0.05	ns	0.68	ns	0.68	ns
	Quinby	-0.88	ns	0.08	ns	1.27	ns	0.13	ns
	Wachapreague	2.17	ns	-0.55	ns	-0.82	ns	-1.02	ns
	York River	1.08	ns	-0.38	ns	—	—	—	—
SC	Hungar's Creek	0.53	ns	2.81	**	-0.37	ns	1.78	*
	North River	-1.38	ns	2.73	***	-1.23	ns	2.91	***
	Quinby	-0.71	ns	2.00	***	-0.07	ns	2.08	***
	Wachapreague	-1.48	ns	1.89	***	-1.49	ns	1.62	**
	York River	-1.08	ns	3.11	***	—	—	—	—
VA	Hungar's Creek	0.45	ns	-1.92	*	-0.95	ns	-2.39	*
	North River	-0.30	ns	-2.70	***	-0.15	ns	-2.80	***
	Quinby	-0.12	ns	-1.10	**	-4.23	*	-1.35	***

continued on next page

TABLE 3.  
continued

		Autumn 2002				Autumn 2003			
		# Live		Length		# Live		Length	
		Parameter	P	Parameter	P	Parameter	P	Parameter	P
	Wachapreague	-0.73	ns	-0.76	*	0.01	ns	-0.68	ns
	York River	0.08	ns	-1.24	†	—	—	—	—

C. Linear contrasts of mid-parent vs. hybrid

		Autumn 2002				Autumn 2003			
		# Live		Length		# Live		Length	
		Parameter	P	Parameter	P	Parameter	P	Parameter	P
Hybrid Cross	Site								
KK × MA	Hungar's Creek	-11.33	ns	-4.21	ns	-7.00	ns	1.19	ns
KK × MA	North River	12.67	ns	3.33	ns	19.00	ns	5.69	*
KK × MA	Quinby	-2.00	ns	3.37	ns	23.67	*	2.61	ns
KK × MA	Wachapreague	-18.33	ns	0.07	ns	-13.00	ns	0.58	ns
KK × MA	York River	-3.33	*	4.17	ns	—	—	—	—
KK × NJ	Hungar's Creek	6.33	ns	3.04	ns	-3.67	ns	-1.83	ns
KK × NJ	North River	-12.00	ns	4.64	ns	-3.33	ns	4.92	*
KK × NJ	Quinby	-1.67	ns	0.82	ns	1.33	ns	-1.63	ns
KK × NJ	Wachapreague	-3.33	ns	-2.83	ns	15.00	—	-3.92	ns
KK × NJ	York River	4.00	ns	1.47	ns	—	—	—	—
KK × SC	Hungar's Creek	-7.00	ns	3.18	ns	-12.67	ns	4.05	ns
KK × SC	North River	-20.00	ns	-2.72	ns	-2.67	ns	0.37	ns
KK × SC	Quinby	2.00	ns	2.44	ns	6.67	ns	-0.34	ns
KK × SC	Wachapreague	-14.67	ns	3.31	ns	21.67	ns	-0.06	ns
KK × SC	York River	1.67	ns	2.26	ns	—	—	—	—
KK × VA	Hungar's Creek	—	—	—	—	—	—	—	—
KK × VA	North River	—	—	—	—	—	—	—	—
KK × VA	Quinby	20.67	**	6.39	*	16.33	ns	1.56	ns
KK × VA	Wachapreague	9.00	ns	6.37	*	0.33	ns	7.23	ns
KK × VA	York River	8.67	ns	7.55	*	—	—	—	—
MA × NJ	Hungar's Creek	-17.00	ns	-0.45	ns	-22.00	ns	5.09	ns
MA × NJ	North River	4.67	ns	4.15	ns	-5.00	ns	1.95	ns
MA × NJ	Quinby	-1.67	ns	2.77	ns	3.00	ns	3.47	ns
MA × NJ	Wachapreague	-13.00	ns	-0.31	ns	2.00	ns	-0.02	ns
MA × NJ	York River	-22.67	*	-4.32	ns	—	—	—	—
MA × SC	Hungar's Creek	-2.33	ns	6.13	ns	-19.67	ns	6.16	ns
MA × SC	North River	9.33	ns	12.70	ns	-4.33	ns	10.48	***
MA × SC	Quinby	4.00	ns	5.69	*	0.33	ns	6.14	**
MA × SC	Wachapreague	5.00	ns	8.34	**	10.67	ns	5.65	ns
MA × SC	York River	-5.00	ns	9.64	**	—	—	—	—
MA × VA	Hungar's Creek	—	—	—	—	—	—	—	—
MA × VA	North River	—	—	—	—	—	—	—	—
MA × VA	Quinby	3.33	ns	7.67	**	0.00	ns	3.73	ns
MA × VA	Wachapreague	-8.67	ns	2.52	ns	1.00	ns	7.45	*
MA × VA	York River	-8.00	ns	12.91	*	—	—	—	—
NJ × SC	Hungar's Creek	-24.67	ns	8.08	ns	-17.00	ns	-0.68	ns
NJ × SC	North River	-2.67	ns	3.21	***	9.33	ns	4.75	*
NJ × SC	Quinby	-11.00	ns	1.26	ns	-9.33	ns	2.85	ns
NJ × SC	Wachapreague	0.00	ns	-0.24	ns	-18.33	ns	-4.60	ns
NJ × SC	York River	-2.33	ns	4.71	ns	—	—	—	—
NJ × VA	Hungar's Creek	—	—	—	—	—	—	—	—
NJ × VA	North River	—	—	—	—	—	—	—	—
NJ × VA	Quinby	7.67	ns	6.98	**	3.00	ns	4.09	ns
NJ × VA	Wachapreague	33.00	*	7.01	**	-1.00	ns	3.33	ns
NJ × VA	York River	8.00	ns	13.34	***	—	—	—	—
SC × VA	Hungar's Creek	—	—	—	—	—	—	—	—
SC × VA	North River	—	—	—	—	—	—	—	—
SC × VA	Quinby	6.67	ns	6.65	**	19.67	ns	4.87	ns
SC × VA	Wachapreague	8.33	ns	5.85	*	-13.00	ns	6.11	ns
SC × VA	York River	0.33	ns	14.23	***	—	—	—	—

ns = not significant, \* =  $P < 0.05$ , \*\* =  $P < 0.01$ , \*\*\* =  $P < 0.001$ , — = not testable.

TABLE 4.

Pairwise correlation coefficients and significance tests among the group means at the five field sites. A. Survival as indicated by the total number of live animals collected in the core samples. B. Shell length. C. Shell width. Field data from the Autumn 2002 samples are below the diagonal and the values above the diagonal represent data from Autumn 2003.

A. Survival					
		Hungar's Creek	North River	Quinby	Wachapreague
Hungar's Creek	r		0.106	0.422	0.359
	P		ns	ns	ns
North River	r	-0.024		-0.055	-0.026
	P	ns		ns	ns
Quinby	r	0.228	0.414		-0.259
	P	ns	ns		ns
Wachapreague	r	0.380	0.434	0.487	
	P	ns	ns	ns	
York River	r	0.397	0.009	0.413	0.707
	P	ns	ns	ns	**
B. Shell Length					
		Hungar's Creek	North River	Quinby	Wachapreague
Hungar's Creek	r		0.838	0.802	0.688
	P		**	**	*
North River	r	0.877		0.895	0.705
	P	***		***	**
Quinby	r	0.921	0.912		0.868
	P	***	***		***
Wachapreague	r	0.807	0.837	0.900	
	P	***	***	***	
York River	r	0.921	0.894	0.954	0.918
	P	***	***	***	***

ns = not significant, \* =  $P < 0.05$ , \*\* =  $P < 0.01$ , \*\*\* =  $P < 0.001$ .

parent strains that we tested—the MA and VA lines, both of which also showed large negative effects for general combining ability. The few positive effects of crossing within strains occurred in lines that also showed better overall performance (KK and SC), indicated by positive general combining ability. Hence, at least in this first hybrid generation, there is an association between the ability of a line to raise the mean growth of any group to which it contributes genetically and the absence of negative effects of crossing within that line. Conversely lines with negative general combining ability for growth also show inbreeding depression as pure strains relative to hybrid lines.

Our data also provide evidence of nonadditive effects of out-crossing in some of our hybrid crosses. As for pure versus hybrid contrasts, midparent contrasts are significant mainly for crosses involving two of the five parental strains, MA and VA. Keeping in mind that these two lines also show the most severe negative impacts of within-line crossing, as indicated by the pure versus hybrid contrasts as well as negative effects in the general combining ability contrasts, the positive nonadditive effects of out-crossing for these strains are attributable less to the superiority of hybrids, than to the poor performance of these two pure lines. That is, whereas hybrid crosses involving the MA and VA line are likely to exceed expectations based on the mean of their parental lines, these crosses are still inferior to hybrid crosses among other parental strains.

Our analysis of midparent comparisons in the hatchery and in the field, however, cannot determine whether they are the result of

dominance or epistasis. Under the dominance hypothesis, hybrid crosses involving the poorest pure lines would perform better than the mean of their parental strains because of dominance of the alleles from the other (better) parent. Alternatively, to the degree to which the parental lines are homozygous at loci involved in epistatic complexes, this first generation of hybrid progeny would be expected to inherit entire gene complexes from their parents; and we would expect, therefore, that out-crossing of the poorest pure strains would also tend to enhance the performance of  $F_1$  progeny by increasing the probability that they possess alleles that interact favorably. Only in the second generation posthybridization would we expect such fixed complexes, if they exist, to be disrupted by recombination (Falconer & Mackay 1996, Lynch & Walsh 1998). It is important, however to note that these nonadditive effects are restricted to the poorest performing parental lines and that there was little indication of nonadditive gene action in line crosses among the better parental lines. Thus, selective breeding and domestication efforts that emphasize selection on additive genetic variance should be effective, and our data provide no reason to believe that more complicated breeding schemes designed to use nonadditive heterotic effects, whatever the mechanism, would be advantageous over the judicious selection or construction of a base population with low inbreeding depression.

Further, the data presented here suggest that the beneficial effects of out-crossing we observed are not likely to be the result of heterozygosity *per se*. If that were the case, we would have expected that out-crossing would have improved performance in all

TABLE 5.

Pairwise correlation coefficients and significance tests among the group means of three adult characters (shell length, width and number of survivors), between these characters and two of the characters measured in the hatchery and nursery (shell length at the seed stage and the area of spat) and between the spat and seed measurements (this reported only once below the diagonal).

A. Pooling all 5 of the field sites. B. For each site separately.

Field data from the Autumn 2002 samples are below the diagonal and the values above the diagonal represent data from Autumn 2003.

A. Sites Pooled					
		Shell Length	Number Live	Seed Length	Spat Area
Shell length	r	—	0.309	0.613	0.122
	P	—	*	***	ns
Number live	r	0.16	—	0.223	0.298
	P	ns	—	—	*
Seed length	r	0.546	0.184	—	—
	P	***	ns	—	—
Spat area	r	0.1	0.212	0.311	—
	P	ns	ns	**	—
B. By Site					
Hungar's Creek					
		Shell Length	Number Live	Seed Length	Spat Area
Shell length	r	—	-0.216	0.728	0.199
	P	—	ns	**	ns
Number live	r	0.05	—	-0.128	0.518
	P	ns	—	ns	ns
Seed length	r	0.784	0.045	—	—
	P	***	ns	—	—
Spat area	r	0.081	0.356	—	—
	P	ns	ns	—	—
North River					
		Shell Length	Number Live	Seed Length	Spat Area
Shell length	r	—	0.157	0.799	0.04
	P	—	ns	**	ns
Shell width	r	0.993	0.166	0.78	0.031
	P	***	ns	***	ns
Number live	r	0.265	—	0.43	-0.148
	P	ns	—	ns	ns
Seed length	r	0.799	0.309	—	—
	P	***	ns	—	—
Spat area	r	0.068	0.037	—	—
	P	ns	ns	—	—
Quinby					
		Shell Length	Number Live	Seed Length	Spat Area
Shell length	r	—	0.557	0.859	0.204
	P	—	*	***	ns
Number live	r	0.525	—	0.686	0.644
	P	*	—	**	*
Seed length	r	0.872	0.662	—	—
	P	***	**	—	—
Spat area	r	0.207	0.549	—	—
	P	ns	*	—	—

continued on next column

TABLE 5.

continued

		Shell Length	Number Live	Seed Length	Spat Area
Wachapreague					
Shell length	r	—	0.243	0.796	0.305
	P	—	ns	***	ns
Shell width	r	0.991	0.225	0.763	0.306
	P	***	ns	**	ns
Number live	r	0.379	—	-0.174	0.172
	P	ns	—	ns	ns
Seed length	r	0.882	0.364	—	—
	P	***	ns	—	—
Spat area	r	0.246	0.392	—	—
	P	ns	ns	—	—
York River					
		Shell Length	Number Live	Seed Length	Spat Area
Shell length	r	—	—	—	—
	P	—	Entire site destroyed by Hurricane Isabel		—
Number live	r	0.176	—	—	—
	P	ns	—	—	—
Seed length	r	0.867	0.26	—	—
	P	***	ns	—	—
Spat area	r	0.266	0.589	—	—
	P	ns	ns	—	—

ns = not significant, \* =  $P < 0.05$ , \*\* =  $P < 0.01$ , \*\*\* =  $P < 0.001$ .

strains by increasing genome-wide heterozygosity. This is clearly not the case for most of the strains we tested. The two best-performing stocks (KK and SC) as well as the NJ strain show no advantages of outcrossing, and the two lines with the poorest performance show the strongest inbreeding depression.

Finally, there is evidence, at least at the level of strain-means we examined here, that growth performance in the field could be reasonably well predicted by measurements taken at an early stage in the culture process. Measurements collected on same-age spat when they were transferred from the upwelling system to nursery bags were essentially uncorrelated with size at the end of one or two growing seasons, a result that agrees with the findings of Hilbish et al. (1993) who found no significant genetic correlation between early larval growth (0–10 days) and shell length at 9 mo of age. However, the significant correlation between the shell length of seed when it was removed from the spat bags and planted into the field plots and adult characters indicate that seed size at a specific age may be a useful predictor of subsequent growth. This implies that the identification of superior seed for planting in the field could potentially be accomplished based on measurements taken under nursery conditions in commercial farms. Also, in the context of a selective breeding effort, it may be possible to identify and cull the most inferior genotypes based on measurements of juveniles, eliminating a great deal of the labor required to rear all of the animals being tested to harvestable size for evaluation.

Further study is required. Especially interesting would be an assessment of the levels of allelic richness and heterozygosity of both the parents and the progeny and an evaluation of whether these change over time as a consequence of selective mortality of more homozygous progeny. In addition, studies of more advanced

hybrid generations are necessary to identify the genetic mechanisms underlying the phenotypic effects we detected.

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## A POPULATION DYNAMICS MODEL OF THE HARD CLAM, *MERCENARIA MERCENARIA*: DEVELOPMENT OF THE AGE- AND LENGTH-FREQUENCY STRUCTURE OF THE POPULATION

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**ABSTRACT** An individual-based model was developed to simulate growth of the hard clam, *Mercenaria mercenaria*, in response to temperature, salinity and food supply conditions. Unique characteristics of the model are that: (1) length and tissue weight are related only by condition index, so that weight, up to a point, can vary independently of length, and (2) age is decoupled from length. Tissue weight changes result from the difference in assimilation and respiration. Changes in hard clam condition are determined from a standard length-weight relationship for average hard clam growth. Changes in hard clam length (growth) occur only when condition index is greater than zero, which happens when excess weight for a given length is attained. No change in length occurs if condition index is zero (mean case) or negative (less weight than expected at a given length). This model structure resolves limitations that accompany models used to simulate the growth and development of shellfish populations. The length-frequency distribution for a cohort was developed from the individual-based model through simulation of a suite of genotypes with varying physiological capabilities. Hard clam populations were then formed by the yearly concatenation of cohorts with partially independent trajectories that are produced by cohort- and population-based processes. Development and verification of the hard clam model was done using long-term data sets from Great South Bay, New York that have been collected by the Town of Islip, New York. The ability to separately track length and age in the simulations allowed derivation of a general mathematical relationship for describing age-length relationships in hard clam populations. The mathematical relationship, which is based on a twisted bivariate Gaussian distribution, reproduces the features of age-length distributions observed for hard clam populations. The parameters obtained from fitting the twisted bivariate Gaussian to simulated hard clam length-frequency distributions obtained for varying conditions yield insight into the growth and mortality processes and population-dependent processes, compensatory and otherwise, that structured the population. This in turn provides a basis for development of theoretical models of population age-length compositions. The twisted bivariate Gaussian also offers the possibility of rapidly and inexpensively developing age-length keys, used to convert length-based data to age-based data, by permitting a relatively few known age-length pairs to be expanded into the full age- and length-frequency structure of the population.

**KEY WORDS:** *Mercenaria mercenaria*, individual-based model, age-length structure, length-frequency distribution, age-frequency distribution

### INTRODUCTION

Hard clam (*Mercenaria mercenaria*) populations in the bays along the south shore of Long Island, New York, have diminished since reaching peak abundance in the 1970s (Schlenk 2000). The definitive cause of this decline has not been demonstrated, but intensive harvesting may have initially reduced clam abundance (Buckner 1984, COSMA 1985) and experimental observations suggest that recent occurrences of the toxic picoplanktonic alga *Aureococcus anophagefferens* (brown tide, Pelagophyceae) may have impacted larval and juvenile survivorship (Bricelj 2000). Hard clam growth rates may have declined relative to past years (Cerrato 2000a), and the same factors slowing growth may have led to decreased gamete production.

During the 1990s other biological and environmental factors, such as temperature, salinity, phytoplankton species and/or concentrations, and predator abundance, may have changed as well. For example, during the 1990s, mean winter water temperature in the Long Island region was 1°C to 3°C warmer than the long-term average (Nixon et al. 2004). A significant predator, the blue crab

(*Callinectes sapidus*), may have recently increased its population levels in this region. Hard clam abundance may have declined enough to adversely affect fecundity (Kraeuter et al. 2005). Thus, the observed reduction in hard clam abundance and the failure of the Great South Bay fishery to recover, despite a reduction in fishing pressure in recent years, likely result from multiple factors.

A mathematical model provides one approach for investigating the relative effect of the many potential factors affecting hard clam populations. The first objective of this study is then to develop a mathematical model that can simulate the growth and population dynamics of the hard clam in response to specified biological and environmental conditions. The model is implemented for Great South Bay, NY where long-term data sets on hard clam abundance and distribution, collected by the Town of Islip, NY, provide calibration and evaluation of the simulations.

An additional important consideration for the Great South Bay ecosystem is development of management strategies that allow rebuilding of the now depleted hard clam stocks. A reliable means for determining population age-length characteristics is an important aspect of developing management strategies because fisheries models, such as virtual population analysis models, depend on age-frequency information for the population (e.g., Hilborn & Walters 1992, Patterson 1998, Clark 1999, Bradbury & Tagart

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2000). The age structure of populations is also important in ecological research, as indicated by the many theoretical and empirical studies that relate age and lifespan to population processes (e.g., Bayne et al. 1983, Hoenig 1983, Connell & Sousa 1983, Bayne et al. 1983, McNamara 1993, Lundberg & Persson 1993, Nunney & Elam 1994).

Population age is usually obtained from an age-length relationship that prescribes the likelihood that an individual of a given length will have a given age, the age-frequency at length, and conversely, the likelihood that an individual of a given age will have a given length, the length-frequency at age (e.g., Kimura 1977, Mohn 2001). For molluscs, aging typically relies on counting of yearly growth bands in shells (e.g., Cerrato 2000b, Goodwin et al. 2001, Richardson 2001) or statoliths (e.g., Lipinski et al. 1998, Richardson 2001). These methods are costly, time consuming, and technically difficult. As a consequence, most empirical studies do not measure the age distribution of all animals sampled from a population or compare age structures in multiple populations. This limits ecological research into the age-length structure of natural populations and the use of sophisticated age-dependent models in fisheries management.

Because of measurement limitations, information on age structure for multiple samples or assemblages typically relies on an age-length key to estimate age frequency from length frequency. Age-length keys can be as simple as mean age-mean length relationships for an individual cohort, normally obtained by fitting a growth model to empirical data (e.g., Tanabe 1988, Smith et al. 1997, Devillers et al. 1998, Millstein & O'Clair 2001). Many factors affect the length frequency of a single cohort over time (e.g., Craig & Oertel 1966). As a result, the age structure of a population derived from many cohorts may not necessarily match that of a single cohort or even a subset of cohorts and the population age and length structure determined using a cohort-based age-length key may not reflect the population as a whole.

Thus, a second goal of this study is to use the hard clam model, which simulates independently the age and length structure of the population, to investigate the age-length characteristics of a population that are produced by variations in environmental and biological processes. These simulations were used to derive a general mathematical function that describes the age-length relationship and its variations in hard clam populations. A general age-length relationship for hard clam populations provides an approach for inferring the processes that produced the population structure.

The hard clam population dynamics model is described in the next section. This is followed by an examination of simulations of hard clam growth and development for a range of environmental conditions and biological processes. Following this, the simulations that provide the basis for derivation of a hard clam age-length relationship are described and the mathematical formulation of the age-length relationship is given. The discussion section summarizes the results within the context of current understanding of hard clam populations.

## MODEL DESCRIPTION

### Hard Clam Model Structure

The hard clam model simulates a single individual with a particular physiology. The model structure is based on the assumption that changes in weight and length of an individual hard clam are related to the condition of the animal. This assumption is implemented by independently calculating hard clam weight and length over time. The weight obtained at any given time is compared with

an average length-weight relationship that was derived for hard clams (described below). The difference between the simulated weight and that obtained from the average curve determines how "fit" the hard clam is at a given time. The length-weight-condition coordinate system allows shell growth to be defined as a function of animal condition rather than as a function of animal weight. Changes in condition dictate whether length can increase. Positive condition supports somatic or reproductive tissue growth and normally shell elongation. Neutral or negative condition does not. This approach ensures that hard clam weight and length remain only partially coupled during a simulation because weight can change without requiring a corresponding change in length. As a result, realistic length-weight combinations cannot be exceeded, but reductions in condition can occur seasonally, as is observed.

Changes in hard clam weight, condition and length are produced by the environmental and physiological processes that control the growth and reproduction of an individual animal (Fig. 1). Ambient temperature, salinity, and total concentration of suspended solids modify filtration rate, which determines food ingestion. Assimilation is determined by the assimilation efficiency and loss to respiration. The apportionment of the resulting net production into somatic and reproductive tissue is determined by temperature, hard clam weight and animal condition. Somatic tissue growth during times of positive clam condition results in an increase in hard clam shell length. Reproductive tissue is formed when net production and condition are positive and temperature is favorable for reproduction. Spawning occurs when the gamete fraction exceeds a threshold fraction of total animal weight. Periods of negative net production result in resorption of reproductive tissue to cover metabolic needs and eventually resorption of somatic tissue.

A cohort is created from the individual-based model by a set of independently-simulated individuals defined by a range of physiological and genetic capabilities (Fig. 2). The frequency of each

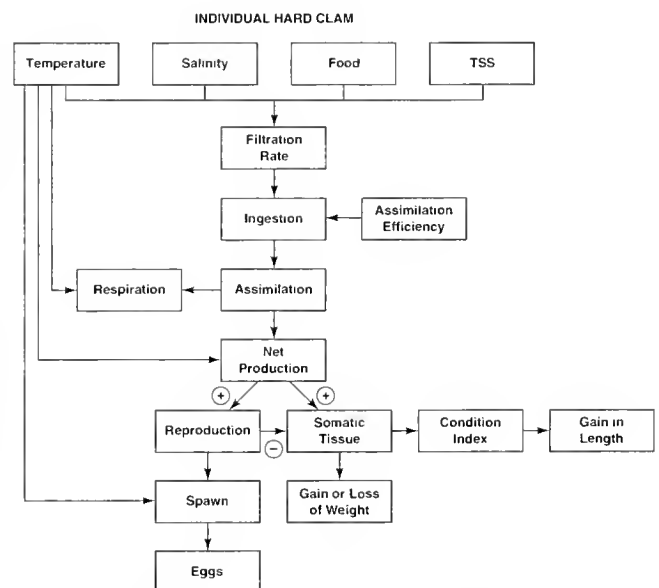


Figure 1. Schematic of the processes and transfers included in the individual-based hard clam model. Total suspended solids is abbreviated as TSS. The division of net production is determined by temperature, animal weight, and animal condition. Positive net production (+) results in formation of reproductive and somatic tissue. Negative net production (−) results in resorption of reproductive tissue.



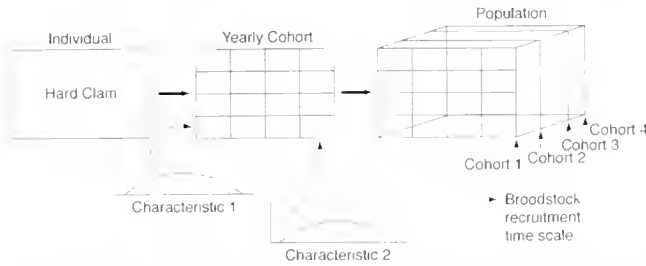


Figure 2. Schematic of the approach used to scale the results of the individual-based clam model to the cohort and population level. For the Great South Bay, NY reference simulation (Figures 10 and 11), characteristics 1 and 2 were the length of a 2-y-old hard clam and assimilation efficiency, respectively. See text for details.

genotype in the cohort is determined by a probability distribution, described below, so that the cohort is a weighted sum of the set of independently-simulated individual genotypes. Concatenations of cohorts over a period of years, as determined by a broodstock-recruitment relationship, builds a hard clam population much as a series of yearly recruitment events and subsequent growth does in the natural world (Fig. 2).

The governing equations used to calculate changes in hard clam weight, condition, and length are described in the following section. This is followed by descriptions of the parameterizations used to represent the physiological processes determining growth and reproduction of an individual hard clam. The final sections describe the approaches used to specify the cohort and population genetics and the broodstock-recruitment relationship used to form the population structure.

#### Hard Clam Model Governing Equations

##### Weight Equation

Changes in hard clam body weight ( $W$ , mg dry wt) over time ( $t$ ) are based on net production, which is given by the difference in assimilation ( $A$ ) and respiration ( $R$ ) as:

$$\frac{dW}{dt} = (A - R) W \quad (1)$$

The weight change obtained from Eq. (1) is the primary input into the calculation of hard clam condition index, described in the next section. The parameterizations for assimilation and respiration for the individual-based hard clam model are described in subsequent sections and definitions and values of coefficients are given in Table 1.

##### Condition Index Equation

Hard clam condition index,  $C(L, W)$ , is defined as a ratio of the current weight,  $W(t)$  obtained from Eq. (1), to a standard weight,  $W_0(L)$ , and a maximum weight,  $W_m(L)$ , of an individual hard clam as:

$$C(L, W) = \frac{W(t) - W_0(L)}{W_m(L) - W_0(L)} \quad (2)$$

where the standard and maximum clam weights are functions of clam length,  $L$  (mm). The condition index obtained from Eq. (2) indicates how fit a clam is at any given time for a specified set of environmental conditions. Positive condition index, when a clam has a mass for a given length that is above the standard value, ranges from 0–1. Values less than zero occur when a clam has a mass for a given length below that obtained from the standard length-weight relationship.

The standard and maximum weights for individual hard clams as a function of clam length used in Eq. (2) are obtained from empirically-derived relationships. The weight and length observations for 30–120 mm hard clams given in Hibbert (1977) were used to develop an allometric equation. However, using this allometric equation for the standard length-weight relationship resulted in growth for 5–20 mm hard clams that was too rapid relative to observed growth rates for this size range. Examination of additional hard clam length and weight data (Krauter unpubl. data), that included 5–20 mm clams ( $n = 450$ ), showed that small hard clams weigh more at a given length than is estimated from the Hibbert (1977) length-weight relationship. Combining the 5–20 mm hard clam data with the Hibbert (1977) data allowed a new average length-weight relationship to be obtained:

$$W_0(L) = a_0 L^3 + b_0 L^2 + c_0 L + d_0. \quad (3)$$

The maximum weight attained for a given length is:

$$W_m(L) = a_m L^3 + b_m L^2 + c_m L + d_m. \quad (4)$$

This length-weight relationship (Fig. 3) allows for small hard clams that are heavier for a given length and large clams that are longer for a given weight than the values obtained from the relationship derived by Hibbert (1977). The implication is that small hard clams do not follow allometric growth. Implementing Eqs. (3) and (4) with the hard clam model resulted in simulated growth rates that gave lengths during the first five years of clam life that matched the hard clam age-length observations given in Wallace (1991).

The time-dependent variation in hard clam condition index (Eq. 2) is calculated from changes in weight (Eq. 1), the average and maximum length-weight relationships (Eqs. 3 and 4, respectively) and length (Eq. 6 in the next section) as:

$$\frac{dC(L, W)}{dt} = \frac{1}{(W_m(L) - W_0(L))^2} \left[ (W_m(L) - W_0(L)) \left( \frac{dW}{dt} - \frac{dW_0}{dL} \frac{dL}{dt} \right) - (W(t) - W_0(L)) \left( \frac{dW_m}{dL} - \frac{dW_0}{dL} \right) \frac{dL}{dt} \right]. \quad (5)$$

##### Length Equation

When excess weight for a given length relative to the average length-weight relationship (Eq. 3) occurs, a positive condition index, the excess weight produces an increase in shell length calculated as:

$$\frac{dL}{dt} = gl(C) L \frac{L_{mf} - L}{L_{mf}} \quad (6)$$

where  $gl(C)$  is the rate of shell length increase and  $L_{mf}$  sets a maximum hard clam length. The form of Eq. (6) results in incrementally smaller increases in shell length as the maximum length is approached.

The rate of shell length change is a function of hard clam condition and is assumed to follow a hyperbolic relationship:

$$gl(C) = gl_{max} \frac{C(L, W)}{glk + C(L, W)} \quad (7)$$

where  $gl_{max}$  is the maximum specific rate of increase in length,  $glk$  is the condition index value at which hard clam length increments at one-half the value of the maximum rate, and condition,  $C(L, W)$ , is obtained from Eq. (2). The rate of shell increase is analogous to the parameter in the von Bertalanffy equation, which determines the rate of length increase ( $k$ ). As a result, observations and models

TABLE 1.

Definitions, units, and values of parameters and coefficients used in the individual-based hard clam model.

Coefficient	Definition	Units	Value
$a_0$	average weight constant	mg dry wt (mm) <sup>-3</sup>	$1.1373 \times 10^{-5}$
$b_0$	average weight constant	mg dry wt (mm) <sup>-2</sup>	$-6.6545 \times 10^{-5}$
$c_0$	average weight constant	mg dry wt (mm) <sup>-1</sup>	$1.4092 \times 10^{-3}$
$d_0$	average weight constant	mg dry wt	-0.014814
$a_m$	maximum weight constant	mg dry wt (mm) <sup>-3</sup>	$1.706 \times 10^{-5}$
$b_m$	maximum weight constant	mg dry wt (mm) <sup>-2</sup>	$-9.9817 \times 10^{-5}$
$c_m$	maximum weight constant	mg dry wt (mm) <sup>-1</sup>	$2.1139 \times 10^{-3}$
$d_m$	maximum weight constant	mg dry wt	-0.02222
$L_{inf}$	maximum length	mm	150
$gl_{max}$	maximum specific rate of length increase	d <sup>-1</sup>	0.008
$glk$	0.5 $gl_{max}$	condition	0.20
$fa$	filtration rate dependence on length	none	0.96
$fb$	filtration rate dependence on temperature	none	0.95
$fc$	constant	(cm <sup>-1</sup> °C ind min) mL <sup>-1</sup>	2.95
$f_0$	filtration rate coefficient	none	0.25
$T_{Low}$	low temperature cut off for filtration	°C	7.5
$T_l$	temperature cut off for filtration	°C	0.5
$T_{High}$	high temperature cut off for filtration	°C	29.0
$f_{s0}$	salinity filtration constant	none	-4.302
$f_{s1}$	salinity filtration	ppt <sup>-1</sup>	0.4144
$f_{s2}$	salinity filtration constant	ppt <sup>-2</sup>	$-8.1027 \times 10^{-3}$
$a_{TSS}$	total suspended solids filtration constant	L (g dry wt) <sup>-1</sup>	1.225
$b_{TSS}$	total suspended solids filtration constant	none	-0.375
$AE_0$	base assimilation efficiency	none	0.075
$AE_l$	assimilation efficiency constant	none	0.575
$AE_k$	assimilation efficiency half saturation coefficient	mg dry wt	0.8001
$a_r$	base respiration rate	μL O <sub>2</sub> (h g dry wt) <sup>-1</sup>	52.1314
$b_r$	respiration weight dependence	none	0.8484
$c_r$	respiration temperature dependence	(°C) <sup>-1</sup>	0.1012
$T_0$	base respiration temperature	°C	20.0
$R_1$	reproductive efficiency constant	(°C) <sup>-1</sup>	1.5
$R_0$	reproductive efficiency constant	none	0.125
$SR_0$	spawn ratio constant	none	0.2
$SR_1$	spawn ratio constant	mg dry wt	3.0
$SR_2$	spawn ratio constant	(mg dry wt) <sup>-1</sup>	0.0346

of hard clam growth (Kennish & Loveland 1980, Loesch & Haven 1973) can be used as a guide for determining the parameters controlling the rate of shell growth in Eq. (7).

#### Model Parameterizations

##### Filtration Rate

Doering and Oviatt (1986) provide a relationship for hard clam filtration rate ( $FR$ ) as a function of clam length and temperature ( $T$ ) of the form:

$$FR_0(L, T) = \frac{L^{fa} T^{fb}}{fc} \quad (8)$$

that allows filtration rate to increase with increasing hard clam length and temperature.

Results from experiments designed to show the effect of temperature on hard clam filtration rate over a range of lengths (Hamwi 1969) indicate that filtration rate is maximal between 17°C and 25°C, decreases at temperatures above and below these values, and ceases at 6°C and 32°C. This pattern holds over a range of hard clam lengths. This temperature dependency was incorporated by modifying Eq. (8) as:

$$FR_1(L, T) = FR_0 f_0 \left( 1 + \tanh\left(\frac{T - T_{Low}}{T_f}\right) \right) \left( 1 - \tanh\left(\frac{T - T_{High}}{T_l}\right) \right) \quad (9)$$

where the hyperbolic tangent function ( $\tanh$ ) provides the temperature dependency that is observed in the observations presented in Hamwi (1969). However, the use of Eq. (9) with an upper temperature limit ( $T_{High}$ ) on maximal filtration rate of 25°C produced incorrect growth rates for simulations that used summer temperatures that exceeded this value. Setting the upper temperature limit for maximum filtration rate to 29°C (Fig. 4) gave correct growth rates for hard clams in environments where the summer maximum temperatures exceeded 25°C. Some evidence for a higher temperature limit on filtration is provided by observations (Ansell 1968) that show hard clam growth up to 28–29°C, which then decreases at higher temperatures. These limited observations and the simulation results obtained using Eq. (9) suggest that a latitudinal variation in the upper temperature limit for hard clam maximum filtration may exist. However, verification studies using environmental conditions from southern regions have not been done. Thus, the form of the filtration rate temperature dependency for temperatures exceeding those typical of Great South Bay is tentative.

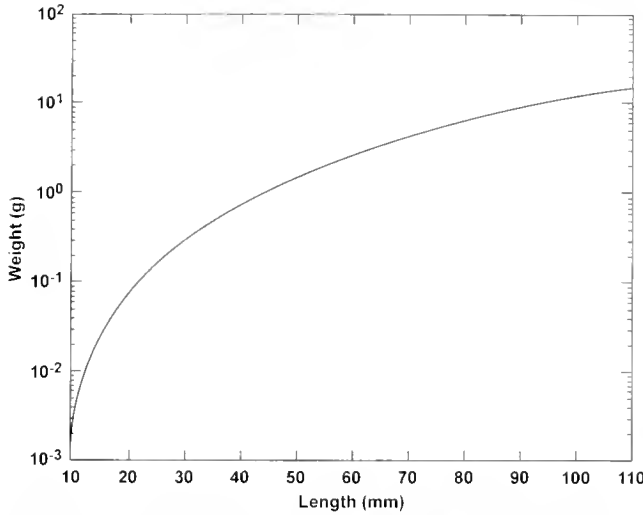


Figure 3. Standard hard clam length-weight relationship given by Eq. (3).

Additional experimental results show that hard clam filtration rate increases with increasing salinity (Hamwi 1969). This effect, described by a quadratic function, modifies filtration rate as:

$$FR_2(L, T, S) = FR_1(f_{s0} + f_{s1} S + f_{s2} S^2) \quad (10)$$

where  $S$  is the ambient salinity.

Increased total suspended solids ( $TSS$ , g dry wt  $L^{-1}$ ) reduce the food acquired by hard clams through reduced filtration and increased pseudofeces production (Bricelj & Malouf 1984, Bricelj et al. 1984, Murphy 1985). The data given in Bricelj and Malouf (1984) and Bricelj et al. (1984) on the percent loss of algae in pseudofeces as a function of sediment concentration were used to develop a relationship that reduces the effectiveness of filtration with increasing  $TSS$  concentration. This effect is added to the filtration rate parameterization as:

$$FR(L, T, S, TSS) = FR_2(1 - a_{TSS} TSS^{b_{TSS}}). \quad (11)$$

The filtration rate given by Eq. (11) is the value that is input to the hard clam model.

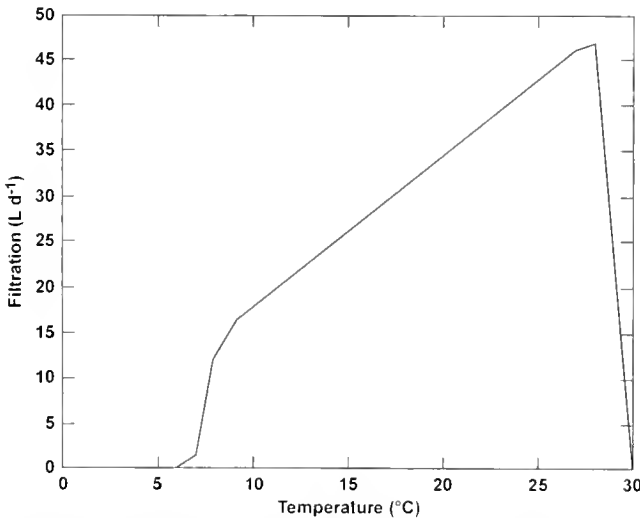


Figure 4. Hard clam filtration rate as a function of temperature calculated using Eq. (9) for a 1 g dry wt clam.

### Assimilation and Assimilation Efficiency

Assimilation ( $A$ ) is determined by:

$$A = FR AE(W) Food(t) \quad (12)$$

where filtration rate ( $FR$ ) is obtained from Eq. (11) and the weight-dependent assimilation efficiency,  $AE(W)$ , determines the fraction of the available food that is assimilated.

The assimilation efficiency consists of a base efficiency ( $AE_0$ ) for clams less than 30 mm. The base assimilation efficiency is low (Table 1) because the filtration (Eq. 8) and respiration (see Eq. 14, later) relationships produce inordinately high growth rates for small hard clams. The low base assimilation efficiency compensates for this imbalance. The implication is that small hard clams have reduced assimilation, either because of lower filtration rates, less efficient filtration or lower assimilation efficiency because of shorter guts (Hughes 1980, Willows 1992). For larger hard clams, the assimilation efficiency increases in a hyperbolic manner with increasing weight as:

$$AE(W) = AE_0 + \frac{AE_1 W}{AE_k + W} \quad (13)$$

where  $AE_k$  is the weight (Eq. 3) of a 42.5 mm hard clam and the asymptotic assimilation efficiency is 0.80. The food time series ( $Food(t)$ ) used in Eq. (12) is described in the Environmental Data Sets section.

### Respiration

Respiration is the primary metabolic loss and is parameterized using a general bivalve relationship obtained at 20°C (Powell & Stanton 1985). This relationship was scaled for temperature effects on respiration by using the  $Q_{10}$  values for hard clam respiration that are summarized in Grizzle et al. (2000). These values were averaged, except for two that fell outside the range of the others, to obtain a  $Q_{10}$  value of 2.75. The respiration relationship is:

$$R(W, T) = a_r W^{b_r} e^{(T-T_0)/T_{10}} \quad (14)$$

which gives increasing respiration rate with increasing temperature (Fig. 5). The respiration rate, in  $\mu L O_2 h^{-1} clam^{-1}$ , was converted to energy equivalent units using  $4.75 \text{ cal } (mL O_2)^{-1}$ ,  $5000 \text{ cal } (g \text{ dry wt})^{-1}$  (Hibbert 1977), and  $4.184 \text{ cal } J^{-1}$ .

### Reproduction

Hard clams are assumed to be sexually mature when they reach 20 mm (Eversole 2000). For hard clams of this length and larger, positive net production is apportioned into somatic and reproductive tissue using a temperature-dependent reproduction efficiency of the form:

$$R_{eff}(T) = R_1 T - R_0 \quad (15)$$

Equation 15 applies over the range of temperatures, reported in Eversole (2000), at which hard clams form reproductive tissue. The temperature-dependent reproductive efficiency, which ranges between zero and 0.9, determines the fraction of net production that goes into reproductive tissue. The coincidence of positive reproductive efficiency and positive condition index, results in the formation of reproductive tissue. At high temperatures, reproductive tissue formation is limited by scope for growth, which is controlled by the rapid drop in filtration rate above 29°C (Eq. 9, Fig. 4) and by the number of days that hard clams experience temperature between 20°C and 27°C, as described in the following section.

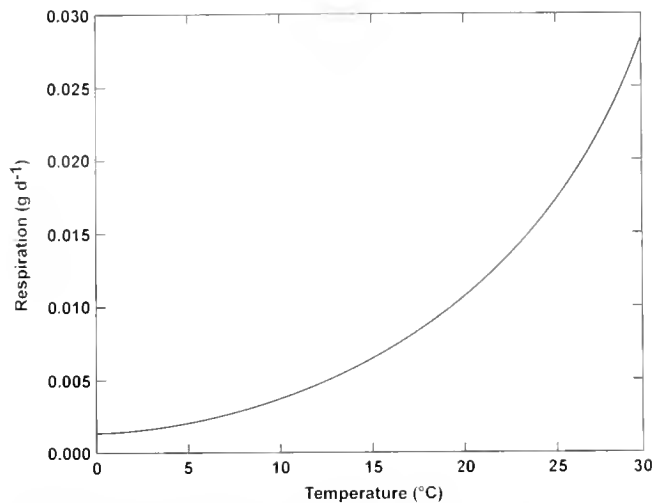


Figure 5. Hard clam respiration rate as a function of temperature calculated using Eq. (14) for a 1 g dry wt clam.

### Egg Production and Spawning

Spawning occurs when reproductive tissue is a certain fraction of total hard clam body weight and condition index is positive. These criteria, which set a spawning threshold, allow spawning to be dynamically determined by environmental conditions and hard clam condition, rather than being imposed.

The ratio of reproductive tissue to total body weight at which spawning occurs was determined by comparing the simulated number of eggs produced by a spawn to observations (Davis & Chanley 1956, Bricelj 1979, Bricelj & Malouf 1980, Malouf 1991, Eversole 2000) that show that an individual hard clam produces 20–40 million eggs in a year. Additional observations (Bricelj & Malouf 1980) show that the number of eggs produced drops as a function of hard clam weight as length exceeds 60–70 mm, which provides a constraint for simulated spawning.

The number of eggs produced in a simulated spawn was obtained by converting the reproductive tissue to eggs using 51 ng dry wt egg<sup>-1</sup> (Eversole 2000). The initial simulations that used a fixed ratio of reproductive tissue to total body weight to trigger spawns overestimated egg production per individual in comparison with observations. Moreover, in some simulations, large hard clams failed to spawn because the scope for growth was inadequate to reach the spawning threshold equivalent to that in the smaller clams. Thus, a spawn ratio (*SR*) that depends on hard clam weight of the form:

$$SR = SR_0 + (W - SR_1) SR_2 \quad (16)$$

was developed to provide the trigger for spawning. For hard clams less than 3 g dry wt, the ratio of reproductive tissue to total body weight at which spawning occurs is 0.2. As hard clam weight increases beyond 3 g dry wt, the spawn ratio decreases, reaching a minimum value of 0.08 for clams of 11 g dry wt. Spawning by larger hard clams is triggered at a lower fraction of reproductive tissue to total body weight, which reduces the number of eggs produced per gram weight for large hard clams (Fig. 6) because the total amount of gamete tissue spawned is smaller, but permits simulated total yearly egg production within the observed range (Eversole 2000).

Hard clams in Great South Bay spawn in June and July, with a small spawn in August (Kassner & Malouf 1982). Initial simula-

tions showed spawning that started at the correct time, but continued into September. The fall spawn resulted in total yearly egg production that was too high relative to observations (Eversole 2000). Observations indicate that spawning of Great South Bay hard clams ceases in the fall, although the processes that terminate spawning have not been identified. Attempts to end fall spawning at the appropriate time that were based on changes in food supply or temperature were unsuccessful. Thus, an *ad hoc* approach was developed that depends on days when the water temperature is between 20°C and 27°C, a temperature range that brackets the optimal range for hard clam spawning. Days with temperatures in this range add one quality day. Spawning occurs for quality days between 1 and 60; no spawn occurs if the cumulative quality days exceed 60. This terminates reproduction prior to the fall temperature decrease. The quality day approach resulted in simulated hard clam growth and reproduction that matched observations from Great South Bay. However, this quality day approach may not apply to lower latitude hard clam populations that are observed to spawn later into the fall.

Unspawned gametes are resorbed in the fall when the temperature declines. During times when the temperature is less than 12°C, the reproductive tissue that is stored as gametes ( $W_{reprod}$ ) is used to support basic metabolic processes at a rate of 0.145 d<sup>-1</sup>, which results in use of one-half of the gametic tissue in 4.5 days.

### Genetic Variations and Development of Cohorts

Growth of individual hard clams varies in response to physiological characteristics and environmental conditions, which are reflected in the yearly cohorts that ultimately produce the population (Fig. 2). These variations come from the population genetic variability. For example, variations can arise from initial length, which determines an initial physiological state for an individual, and from differences in physiological responses, such as assimilation rate and respiration rate, which influence growth efficiency.

The genetically-determined variability ( $GV_{i,j}$ ) of a cohort was obtained using a Gaussian function of the form:

$$GV_{i,j} = w_0 e^{-\frac{(C1_i - C1_0)^2}{2\sigma C1^2}} e^{-\frac{(C2_j - C2_0)^2}{2\sigma C2^2}} \quad (17)$$

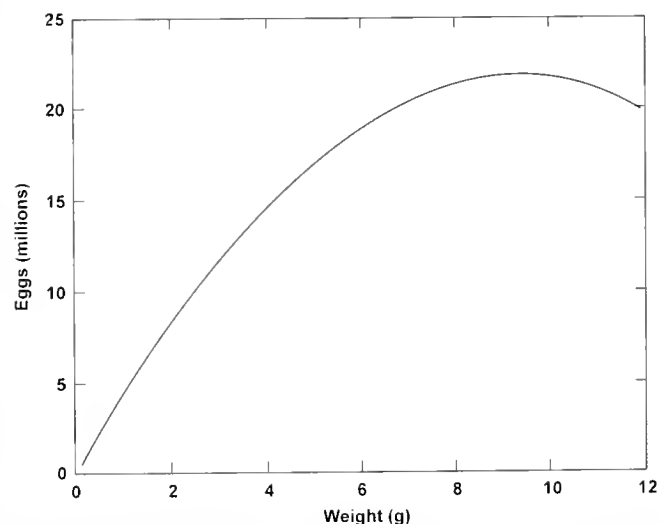


Figure 6. Number of eggs produced as a function of hard clam weight calculated using Eq. (16).

TABLE 2.

Definitions, units, and values of parameters and coefficients used in calculations of the cohort and population structure.

Coefficient	Definition	Units	Value
$w_0$	initial clam density	ind m <sup>-2</sup>	1
$CI_i$	characteristic 1 initial length variation	mm	specified
$CI_0$	characteristic 1 initial length	mm	26
$\sigma_{C1}$	characteristic 1 standard deviation	mm	6.0
$C2_j$	characteristic 2 assimilation rate variation	d <sup>-1</sup>	specified
$C2_0$	characteristic 2 assimilation efficiency mean value	d <sup>-1</sup>	1.0
$\sigma_{C2}$	characteristic 2 standard deviation	d <sup>-1</sup>	0.3
$imax$	maximum range characteristic 1	none	specified
$jmax$	maximum range characteristic 2	none	specified
$Sz_0$	smallest initial length	mm	12.0
$Sz_n$	largest initial length	mm	50.0
$Mr_0$	smallest assimilation efficiency value	d <sup>-1</sup>	0.25
$Mr_n$	largest assimilation efficiency value	d <sup>-1</sup>	1.75
$Ra$	broodstock-recruitment constant	ind g <sup>-2</sup>	$-3.26 \times 10^{-3}$
$Rb$	broodstock-recruitment constant	ind g <sup>-1</sup>	0.0863
$Rc$	broodstock-recruitment constant	ind m <sup>-2</sup>	-0.00228
$B_{0,0}$	egg survival constant	(mg dry wt) <sup>2</sup> g <sup>-1</sup>	-0.1192
$A_{0,0}$	egg survival constant	none	$3.3119 \times 10^{-8}$
$m_1$	mortality rate constant	d <sup>-1</sup>	0.032
$m_2$	mortality rate constant	age <sup>-1</sup>	-1.5201
$m_3$	mortality rate constant	d <sup>-1</sup>	$1.8916 \times 10^{-4}$
$m_4$	mortality rate constant	age <sup>-1</sup>	0.19016

where  $C1$  and  $C2$  represent the characteristics that are used to specify the genetic variability. The Gaussian distribution was centered about the mean value for each genetic characteristic and the range in variation for each was set by the standard deviation ( $\sigma_{C1}$ ,  $\sigma_{C2}$ ) about the mean value (Table 2). The range in variation associated with a particular characteristic is given by  $i$  and  $j$ . The Gaussian weight is normalized so that

$$\sum_{i=1}^{imax} \sum_{j=1}^{jmax} GV_{ij} = 1 \quad (18)$$

Equation 17 gives the cohort distribution of two characteristics. It can be expanded to include as many characteristics as are relevant to describing variations in the population.

The estimate of the range of variability in individual hard clams was obtained from data sets that give the range of lengths of hard clams for cohorts at age (Peterson et al. 1983, Grizzle 1988, Kenish unpublished data on 583 hard clams 0.5–10 y in age). These data were normalized by age and used to derive a relationship that relates the magnitude of the length range to age (Fig. 7). The normalized range suggests a two standard deviation variation in hard clam length at age. This was used as the basis to set the values of  $\sigma_{C1}$  and  $\sigma_{C2}$  in Eq. (17) to give a two standard deviation variation in particular clam characteristics about a mean characteristic value.

Equation 17 was implemented by allowing initial length, assimilation efficiency, and respiration rate to vary. Varying assimilation efficiency is equivalent to varying any process that affects assimilation, including filtration and ingestion rate. Variation in initial length ( $Sz$ ) was introduced by:

$$Sz(i) = Sz_0 + 2(i-1) \text{ for } i = 1, Sz_n \quad (19)$$

where  $Sz_0$  and  $Sz_n$  represent the smallest and maximum initial lengths, respectively. Variation in metabolic rates ( $Mr$ ) was introduced by:

$$Mr(j) = Mr_0 + 0.05(j-1) \text{ for } j = 1, Mr_n \quad (20)$$

where  $Mr_0$  and  $Mr_n$  represent the minimum and maximum range for a particular metabolic process. The initial and maximum values used for variation in length and assimilation efficiency are given in Table 2. Some combinations of initial length and metabolic rate will be less common in a given hard clam cohort and other combinations will be less viable overall either because of metabolic imbalances or metabolic inefficiencies. The surviving individuals

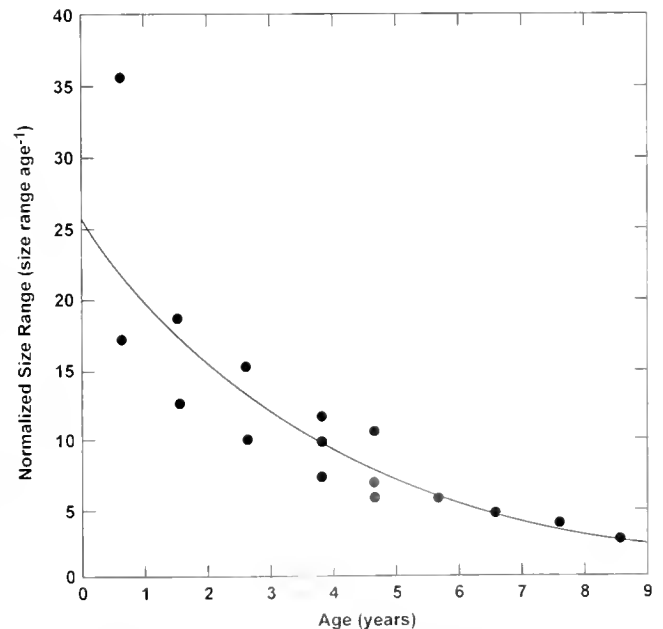


Figure 7. The magnitude of the length range for a hard clam cohort, standardized to cohort age, as a function of cohort age.

determine the genetic structure of the cohort. A cohort is produced in each year of the simulation and the yearly cohorts concatenated to produce the overall population structure (Fig. 2), as described in later sections.

### Mortality

Simulations were started with 2-y-old hard clams because verification data from Town of Islip, NY stock assessments did not include quantitative counts of younger clams. The postsettlement mortality for the hard clams in the first and second years was absorbed into the recruitment calculation, which is described later. The mortality associated with the two-year and older postsettlement hard clam populations is assumed to be from natural processes, such as predation (Malinowski 1985). Mortality from starvation, temperature, and salinity are considered to be small in comparison and are not included as explicit losses. Combined mortality observations from field estimates and transplanted hard clams (Hibbert 1977, Kennish 1978, Buckner 1984, Walker 1983, Kraeuter unpubl. data) show that the specific mortality rate is higher for young clams, lessens for intermediate age clams, and increases for old clams. These data were used to develop daily specific mortality rates ( $M$ ) as a function of hard clam age ( $CA$ ) as:

$$M(CA) = m_1 e^{m_2 CA} \text{ for age } < 3 \quad (21)$$

and

$$M(CA) = m_3 e^{m_4 CA} \text{ for age } > 3 \quad (22)$$

where parameter definitions and values are given in Table 2. The daily specific mortality rates were then used to calculate the fraction of the population that survives ( $SF$ ) to the next year as:

$$SF = 1 - M(CA) T_{yr} \quad (23)$$

where  $T_{yr}$  is the number of days in a year. The survival fraction obtained from Eq. (23) is highest for 2-to-4-y-old hard clams and decreases with age (Fig. 8).

### Environmental Data Sets

#### Temperature and Salinity

Several time series of monthly temperature observations are available for Great South Bay. The year-long time series of monthly temperature observations for 1977–1978 (Bricelj 1979) was used for this study because of its completeness and because it appeared to best represent average conditions (Fig. 9A). The seasonal variation in temperature is as expected, with maximum temperatures in summer and minimum values in winter.

The limited available salinity data for Great South Bay (Quaglietta 1987) indicate that salinity variations are small, with maximum values occurring in late summer and fall. The observed salinity variation of Great South Bay is always near optimal levels for hard clams. These data were used to construct an idealized year-long salinity times series (Fig. 9B) in which salinity varies sinusoidally from a minimum of 22.5 to a maximum of 27.5 with the minimum occurring at year-day 100 (April 10).

#### Chlorophyll $a$ and Food

Chlorophyll  $a$  concentrations for Great South Bay were measured during 1985 at weekly to monthly intervals, except for January, February and March (Quaglietta 1987). The three months for which data were not available were obtained by linear interpola-

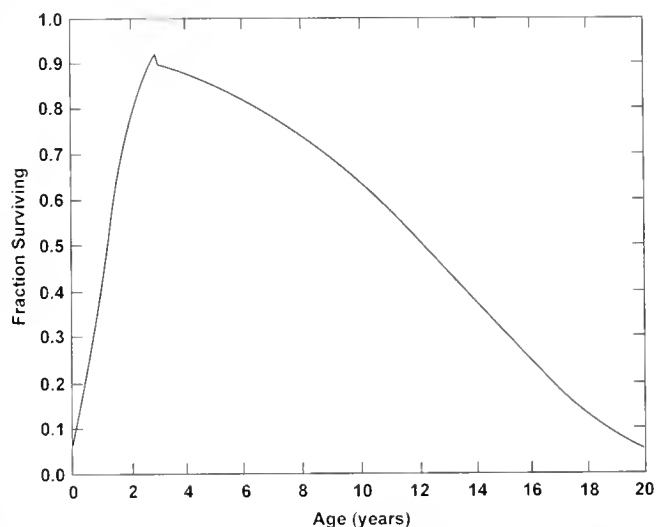


Figure 8. Fraction of hard clams surviving as a function of clam age obtained from Eqs. 21–23. The change in slope of the curve at three years is produced by joining of the two mortality functions.

tion from the nearest measurements. The resultant chlorophyll  $a$  time series (Fig. 9C) shows low chlorophyll  $a$  from January to late April, after which concentrations about double as the spring bloom develops. Chlorophyll  $a$  values during the late summer are reduced from these maximum values and then increase again in late fall (Fig. 9C).

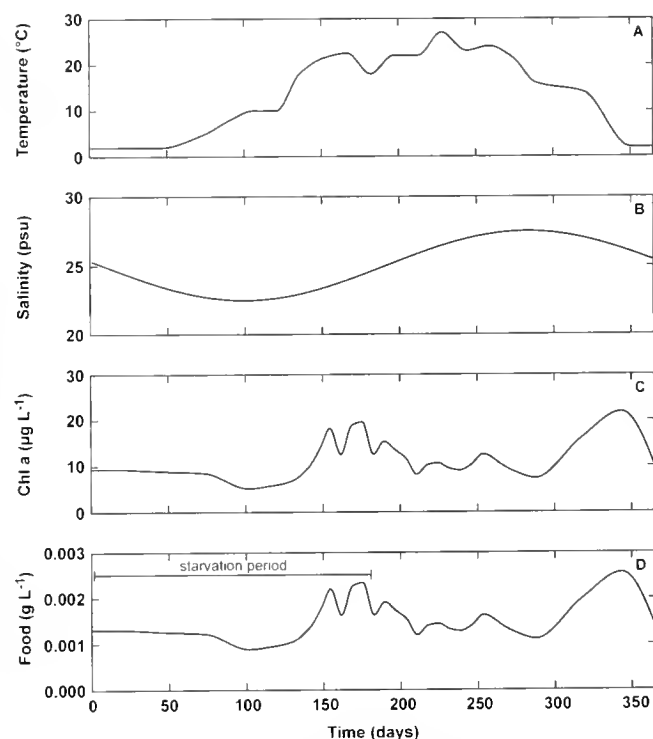


Figure 9. Time series of (A) temperature, (B) salinity, (C) chlorophyll  $a$ , and (D) food, calculated from chlorophyll  $a$  using Eq. (24), that were input to the individual-based hard clam model. The solid line on panel D indicates the time and duration of an imposed starvation period used for other simulations, as described in the text.

Chlorophyll *a* concentration (*chla*) was converted to food (Fig. 9D) using the relationship given in Soniat et al. (1984):

$$\text{Food}(t) = af + bf \text{chla}(t) \quad (24)$$

which includes a nonalgal food supply (*af*) that increases available food above that estimated from chlorophyll *a* and sets a lower limit on the food available to hard clams (Table 3). The importance of a nonalgal food source for marine bivalves has been established by independent measurements of protein, lipid, and labile carbohydrate in a number of bays and estuaries (Soniat & Ray 1985, Soniat et al. 1998, Hyun et al. 2001). The converted food time series (Fig. 9D) was input into the hard clam model via Eq. (12). The food time series resulted in simulated hard clam growth rates that match those reported in Wallace (1991).

#### Total Suspended Solids

A wide range in concentration of suspended solids (2–167 mg L<sup>-1</sup>) has been reported for Great South Bay, with increased values associated with periods of high winds or runoff (Dennison et al. 1991). Monitoring activities during the summer of 1980 by Suffolk County, NY showed that suspended solids values usually are below 20 mg L<sup>-1</sup> (Dennison et al. 1991). Additional limited measurements of particulate organic matter for Great South Bay (Bricelj et al. 1984) and total seston for Long Island Sound (Evjen 1985) suggest an average concentration of 10 mg L<sup>-1</sup>. Increases above this value occur in late fall and winter, which is at a time when clam filtration is reduced. The variation in summer values does not exceed this average value (Evjen 1985). Thus, the average total suspended solids concentration was used in the hard clam filtration relationship given by Eq. (11).

#### Hard Clam Model Implementation

##### Solution Method

The individual-based hard clam model consists of first-order ordinary differential equations that calculate the time-dependent rate of change of weight, length and condition. The dependence among these variables is such that only two are independent and the third can be obtained as a diagnostic calculation. In this implementation of the model, the change in condition and length are obtained from Eqs. (5) and (6), respectively, and are used to calculate weight from Eq. (1).

The set of differential equations is solved with a 5-stage, 4<sup>th</sup>-

order, compact storage Runge-Kutta solution scheme (Carpenter & Kennedy 1994). This solution procedure has truncation errors proportional to the time step to the fourth power making them accurate in time. The procedure has the further benefit of requiring that only two (instead of the more usual 4) intermediate solutions be saved.

#### Initial Conditions and Forcing

The individual-based model was initialized with a two-year-old clam using a range of sizes established by Eq. 19. As a result, simulation year 0 starts with a 2-y-old clam. Conversion of simulation year to actual clam age is done by adding two years. The hard clam simulations are run for 20 y with a time step of 1 day. The individual-based simulations were combined to form populations with one or more cohorts. A 20-y simulation is sufficient to develop a stable cohort and population structure.

The monthly-averaged environmental data sets input to the model were interpolated to daily values. The annual time series for each environmental variable (Fig. 9) is repeated in each year of the 20-y simulation. The simulated weight, length, and condition and additional diagnostic properties, such as egg production, are saved at 10-day intervals. Model calculations are done in terms of J (g dry wt)<sup>-1</sup>.

#### Cohort and Population Calculations

A cohort is constructed from the individual-based model results and is composed of individual hard clams that recruit in the same year. The number of individuals m<sup>-2</sup> in a cohort at each combination of genetic variability (*N<sub>i,j</sub>*) is calculated as:

$$N_{i,j} = N_0 GV_{i,j} \quad (25)$$

where *N<sub>0</sub>* is the initial number of individuals m<sup>-2</sup> in the cohort (Table 3) and *GV<sub>i,j</sub>* is calculated from Eq. (17). All of the cohort calculations used in this study started with an initial value of 1 ind m<sup>-2</sup>, which is consistent with observed hard clam abundances in Great South Bay. The initial value is then apportioned among the various genetic combinations obtained by application of Eq. (17).

Age-dependent mortality, obtained from Eqs. (21, 22), is applied to the cohort and the annual survival fraction (Eq. 23) is used to calculate the reduction in total number of clams in a cohort in a given year as:

$$N_{i,j}(\text{year}) = SF \times N_{i,j}(\text{year}) \quad (26)$$

The total cohort biomass m<sup>-2</sup> (*CB*) is then obtained by summing the product of the number of hard clams associated with each genetic characteristic and the corresponding weight that each has in the month of June (*JW<sub>i,j</sub>*):

$$CB = \sum_{i=1}^{\max} \sum_{j=1}^{\max} N_{i,j} \times JW_{i,j} \quad (27)$$

The simulated June weight is used in the above calculation because this corresponds to the maximum (prespawn) weight for an individual hard clam.

The population biomass in a given year (*PB(year)*) is determined by summing across all cohorts

$$PB(\text{year}) = \sum_{k=1}^{Cmax} CB_k \quad (28)$$

where *k* represents the number of cohorts and *Cmax* is the maximum number of cohorts. The total population biomass obtained

TABLE 3.

Definitions, units, and values of parameters and coefficients used in implementation of the hard clam and the cohort and population calculations.

Coefficient	Definition	Units	Value
<i>a<sub>f</sub></i>	non-algal food concentration	mg dry wt L <sup>-1</sup>	0.520
<i>b<sub>f</sub></i>	food constant	(mg dry wt L <sup>-1</sup> ) (μL L <sup>-1</sup> ) <sup>-1</sup>	0.088
<i>N<sub>0</sub></i>	initial number of clams	ind m <sup>-2</sup>	1
<i>bf<sub>0</sub></i>	cohort biomass reduction	none	6.3258 × 10 <sup>-10</sup>
<i>bf<sub>1</sub></i>	cohort biomass reduction coefficient	(mg dry wt) <sup>-1</sup>	2.3659 × 10 <sup>-11</sup>

from Eq. (28) provides the spawning stock biomass that is used to calculate the number of surviving eggs (Eq. 30), as described below.

#### Population Recruitment

The number of eggs spawned provides the potential recruits that produce a cohort in a given year. Simulated egg production for the spawning stock cannot be converted simply into recruitment, however. A broodstock-recruitment relationship derived for Great South Bay hard clam populations suggests that survivorship varies nonlinearly as a function of broodstock biomass. This relationship was developed using data collected by the Town of Islip, NY, from 1978–2000 expressed as the number of 2-y-old clams in a given year as a function of broodstock biomass two years previously. This relationship is of the form:

$$R(\text{year}) = Ra \times PB(\text{year} - 2)^2 + Rb \times PB(\text{year} - 2) + Rc \quad (29)$$

where  $R(\text{year})$  is the number of 2-y-old clams  $\text{m}^{-2}$  in a given year and  $PB(\text{year} - 2)$  is the spawning stock biomass in  $\text{g m}^{-2}$  that existed two years previously. Krauter et al. (2005) provide additional information on the relationship between recruitment and broodstock in Great South Bay hard clam populations.

The relationship given by Eq. (29) results in a linear increase in recruits with increasing broodstock biomass up to a certain biomass, after which recruits level off as biomass increases, a process known as compensation. Compensatory processes involve cannibalism of young (e.g., Hunt et al. 1987) or competition for food among adults (Frechette & Lefavre 1990, Honkoop & Bayne 2002), which may reduce fecundity. A nonlinear dependency of spat density on spat survival, which may arise from variable predation rates over a range of clam densities (Peterson et al. 1995, Boulding & Hay 1984), would also produce a compensatory broodstock-recruitment pattern.

The broodstock-recruitment relationship given by Eq. (29) does not allow variations in recruitment in years when environmental conditions increase or restrict fecundity or survivorship for a given broodstock biomass. Therefore, the broodstock-recruitment relationship was used with an empirically-derived relationship between clam biomass and fecundity to determine egg survivorship. The biomass-fecundity relationship was obtained by first creating an average population length-frequency distribution from the Town of Islip hard clam data. Simulations using average environmental conditions for Great South Bay (described in the Environmental Data Sets section) were used to obtain hard clam egg production values for a wide range of hard clam lengths and weights. These egg-production values were prorated across the average length-frequency distribution to create a population average fecundity and the fecundity value was normalized to a broodstock value of  $1 \text{ g dry wt m}^{-2}$ . The  $1\text{-g dry wt m}^{-2}$  value was then expanded across the range of anticipated population biomasses obtained from the broodstock-recruitment curve, with the assumption that the source of compensation in the biomass-recruitment curve is not adult competition for resources. In Great South Bay, average hard clam biomass does not normally exceed about 3 large clams  $\text{m}^{-2}$  and this density is unlikely to create conditions limiting clam ingestion, thereby producing competition for resources.

The clam biomass-fecundity relationship was used with the estimate of the number of two-year-old recruits obtained from the broodstock-recruitment curve for a given population biomass to obtain the average egg survivorship to a 2-y-old clam for a range

of population biomass levels. The resulting relationship describing the fraction of eggs surviving to become two-year-old clams ( $S_{2yo}$ ) as a function of population biomass is of the form:

$$S_{2yo} = A_{2yo} e^{I_{2yo} PB(\text{year}-2)} \text{Eggs} \quad (30)$$

where parameter definitions and values are given in Table 2. Eq. (30) gives decreasing survivorship with increasing hard clam biomass, thus maintaining the general compensatory relationship defined by the broodstock recruitment curve, while also allowing conditions of inherently higher fecundity to result in inherently higher recruitment.

A hard clam population is constructed from the yearly cohorts. In each year, a new cohort enters the population with a number of individuals and size structure as described by Eq. (19) and (30). As a consequence, population age frequency and length frequency are independent characteristics, length being derived from the growth of each individual genotype in each cohort and age by the time of cohort birth.

## RESULTS

### Individual Hard Clam Simulations

#### Individual Hard Clam Reference Simulation—Basic Characteristics

The simulated length and weight change of an average individual hard clam (Fig. 10) followed the normal pattern, with increases in weight being larger for smaller-sized clams and length increases being greater for larger clams. Decreases in weight, such as the one that occurs at about 40 mm, result from spawning. For Great South Bay environmental conditions, the simulated length and weight remain above the standard curve, except when spawning occurs, and never exceed the maximum length and weight allowed for hard clams.

The simulated hard clam dry weight (Fig. 11A) increases during the first five simulation years, after which the rate of increase in dry weight declines and levels out at 4–5 g. The short-term decreases in weight are associated with spawning, which is most frequent in simulation years 2 and 3 (hard clam years 4 and 5). Short-term fluctuations in weight decrease in the later years of the

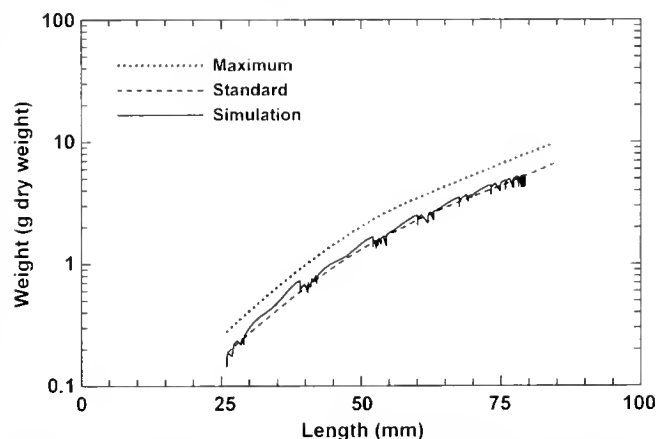


Figure 10. Simulated hard clam length and weight obtained from the reference simulation with the individual-based model. The standard and maximum hard clam length-weight curves obtained from Eqs. 3 and 4, respectively, are shown for comparison. Decreases in simulated hard clam weight at a given length are associated with spawning events or times of negative scope for growth.



simulation as the hard clam gets larger and older and spawns less frequently. The weight decrease that begins in winter and extends into the spring of each simulation year results from reduced food (Fig. 9D) during this time. The simulated change in hard clam length (Fig. 11A) is rapid in the first five simulation years, after which length increases slowly to a maximum value of about 79 mm. The periods of constant length in each simulation year coincide with periods of weight loss, such as occurs during the winter and spring.

The reference simulation is for a hard clam with an average genotype for the cohort as defined by Eqs. (19) and (20). As a consequence, some members of the cohort will grow slower and others faster than this average animal. Likewise, some spawn more and others less frequently. This is then a representative hard clam genotype for the cohort, but not the only genotype. The simulated condition index (Fig. 11B) of this average hard clam ranges between 0.4 and -0.4. Positive condition corresponds to times of increase in weight and shell length and occur mostly during the

first five simulation years. Positive condition is also associated with spawning (Fig. 11A) after which condition declines, normally becoming negative. Periods of negative condition also occur during times of reduced food availability, such as in winter (Fig. 9D). At these times, weight decreases as the hard clam resorbs tissue to cover metabolic needs and length remains constant (Fig. 11A). After simulation year 5, the variability in condition index is reduced (Fig. 11B) because of less frequent spawning. After the first five simulation years (clam age of 7 y) the trend in condition index is negative, with only occasional short-duration periods of positive condition. Condition index remains negative after simulation year 9 (clam age of 11 y).

The negative condition characteristic of the latter stages of hard clam development is expected. Growth slows with increasing age because the time-integrated amount of energy assimilated begins to be balanced by the losses to respiration and reproduction. For the simulated shell growth to slow, as observed at old age, condition index must rarely rise above zero, as positive condition leads to shell growth. The expected rise and fall of condition index with the yearly gametogenic cycle (Fig. 11B) differs from the overall trend in relative condition over the animal's lifetime that describes the decline in growth efficiency as animal size increases (Thompson & Bayne 1974, Hawkins & Bayne 1992, Brown et al. 1993, Lika & Nisbet 2000). This constraint in energy apportionment is also seen in spawning. Spawning is a frequent and regular event during the first four simulation years (clam age of 2–6 y) of the representative animal and spawning season spans much of the summer (Fig. 12A). The number of eggs spawned increases during the first four simulation years (Fig. 12A). In subsequent years, spawning normally occurs once yearly and the number of eggs produced is relatively constant.

#### Individual Hard Clam Reference Simulation—Sensitivity Studies

Exposure to a six-month period without food (Fig. 9D), beginning at the start of simulation year 3, produces starvation and results in a cessation of hard clam growth (Fig. 11A), reduced spawning frequency and egg production (Fig. 12B), and an extended period of negative condition index (Fig. 13). Once food is again available, length increases (Fig. 11A). Spawning frequency and egg production in the following two simulation years (years 4 and 5) is more frequent than observed in the nonstarved simulation (Fig. 12A,B). An additional starvation period in simulation year 6 again results in cessation of growth but the final hard clam length after 10 y of simulation is similar to that obtained from the simulation in which food was available at all times. This rebound effect is well known (e.g., Engle & Chapman 1953, Kobayashi et al. 1997) and originates from the inherently higher growth efficiencies of smaller animals that allows them to appear to "catch up" in growth when energy limitation ceases. Again, spawning frequency and egg production are reduced by starvation (Fig. 12B). Although starvation events do not seem to have a long-term effect on hard clam growth, as indicated by length, there is an effect on reproductive capacity (Fig. 12B).

An assumption made in the development of the individual-based model is that small hard clams have lower assimilation efficiency (Eq. 13). The importance of this assumption is demonstrated by a simulation in which the assimilation efficiency of small hard clams was not reduced. In the first 0.5 y of the simulation, when individuals are less than 30 mm, condition index exceeds 0.5 (Fig. 13), a value well above the condition index

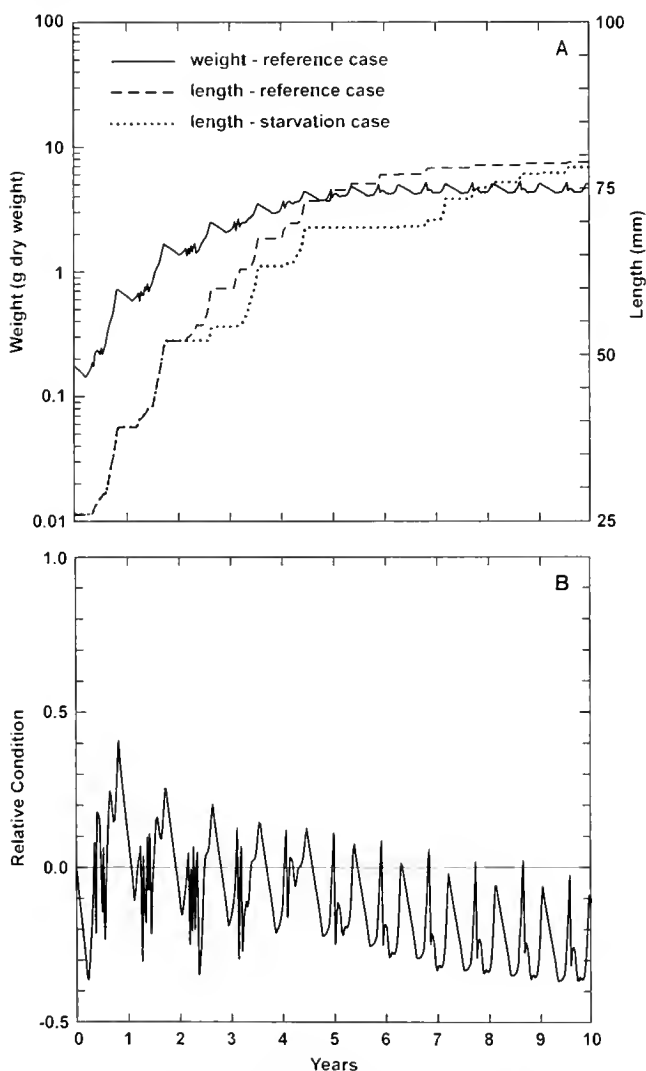


Figure 11. Simulated time development in (A) hard clam weight and length obtained from the reference simulation and a simulation that included a starvation period (cf. Figure 9D) and (B) condition index obtained from the reference simulation. Note that weight and length are given on log and linear scales, respectively.

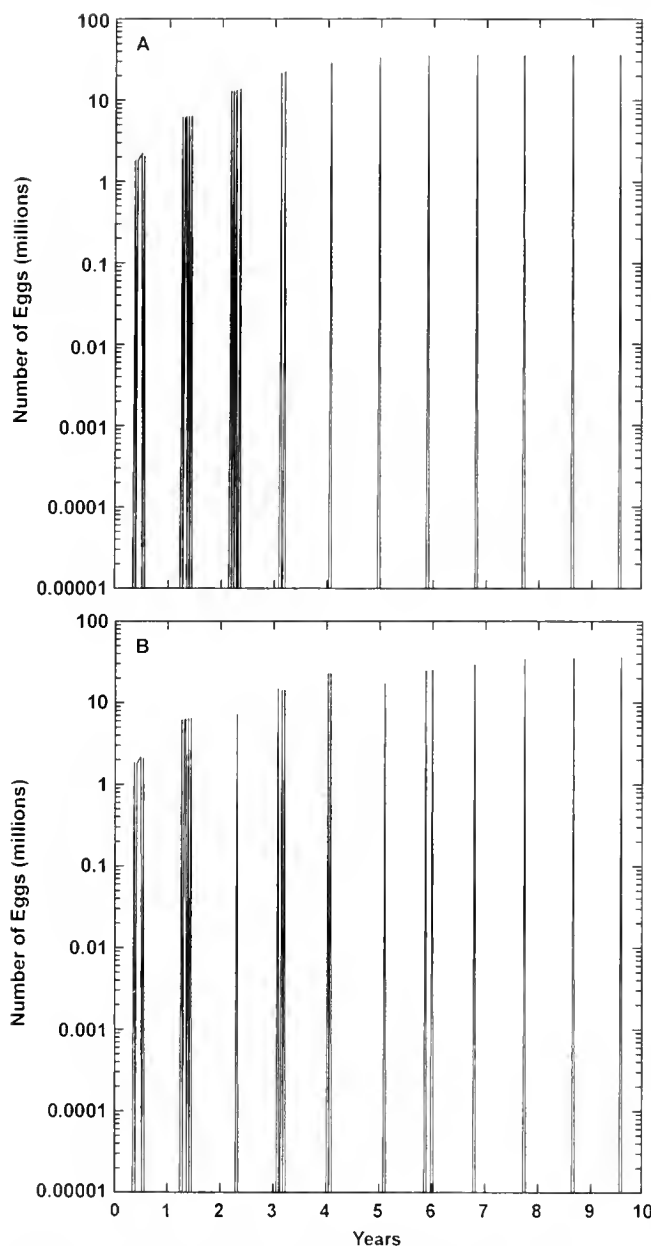


Figure 12. Simulated change in the timing and magnitude of the number of eggs spawned by hard clams exposed to (A) standard food conditions and (B) standard food conditions with an imposed starvation period (Figure 9D).

obtained from the simulation that assumed reduced assimilation efficiency (Fig. 11B). The resultant growth rate for small hard clams is unrealistic.

#### Hard Clam Cohort Simulations

##### Hard Clam Cohort Reference Simulation—Basic Characteristics

The individual hard clam simulations were combined via Eq. (17) to produce a cohort with genetic variation imposed through variability in assimilation efficiency and initial length. The length-frequency distribution of the cohort produced from the individual-

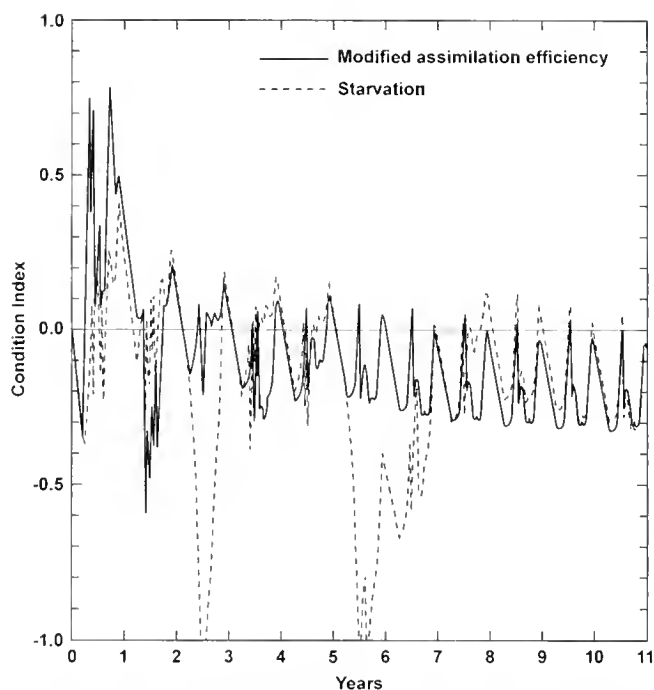


Figure 13. Simulated change in hard clam condition index that occurs in response to relaxing the constraint of reduced assimilation efficiency for small clams (Eq. 13) and to imposing a starvation period (Figure 9C).

based reference simulation (Fig. 14A) shows an initial large peak at 25 mm that decreases in amplitude as the cohort ages and mortality has an effect. The range of lengths included in the cohort increases with age. For example, the cohort in simulation year 4 (clam age year 6) ranges from about 30 mm to almost 75 mm. This expansion of cohort length range with age is well described (Craig & Oertel 1966, Powell et al. 2005).

Cohort biomass (Fig. 14B) begins to increase in simulation year 2, peaks in simulation years 4 and 5 (clam age years 6 and 7) as growth exceeds mortality, after which it decreases to low levels in simulation year 15 and beyond. Cohort egg production is highest in simulation years 2–5 (clam age years 4–7), and declines in later years (Fig. 14B). Much of this trend originates from the increase in fecundity of young clams as they grow and the decline in cohort fecundity with age, as the clams die. The number of survivors in the cohort declines over the simulation, reaching essentially zero after simulation year 12 (Fig. 14B).

##### Hard Clam Cohort Reference Simulation—Sensitivity Studies

The effect of reduced and increased range in genetic variability on cohort structure was tested. Genetic variability was modified by changing the values of  $\sigma_{C1}$  and  $\sigma_{C2}$  in Eq. (17): smaller values yield a restricted range and larger values give an expanded range. The cohort structure produced by a restricted range of genetic variability (Fig. 15A) is similar to that obtained from the basic simulation (Fig. 14A), but with an unrealistic reduction in the spread in length within the individual cohorts. The number of individuals in the modal size class of the cohort is larger than that obtained in the reference simulation because the narrow range of genetic variability retains more individuals in a narrower range of length classes. The expanded range of genetic variability results in

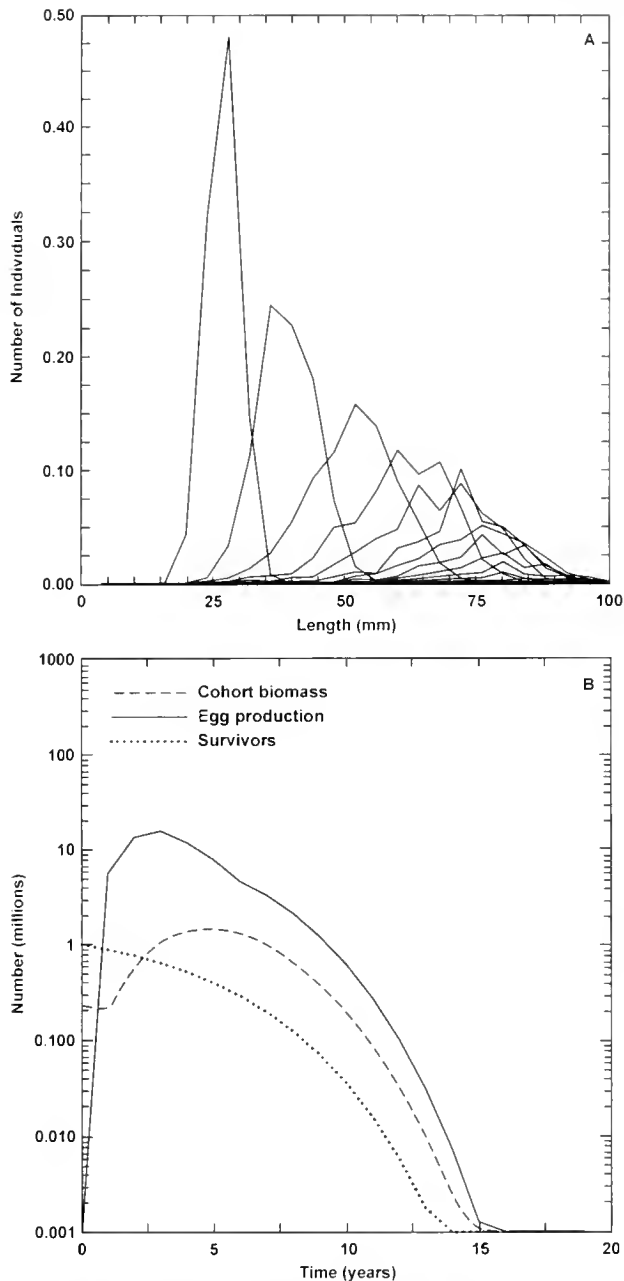


Figure 14. Simulated (A) length-frequency distribution and (B) time development of cohort biomass, egg production, and recruitment constructed from results of individual hard clam simulations subject to the standard environmental conditions (Figure 9) and standard genetic range for initial length and assimilation efficiency (Table 2).

considerable, and again unrealistic, spreading of the cohort (Fig. 15B). Cohorts for simulation years 4 and higher (clam age years 6 and higher) essentially include animals of all lengths.

Respiration rate is another physiological characteristic that produces genetic variability in hard clams. Variation in respiration rate and initial length produces a cohort structure (Fig. 16A) that is similar to that obtained using assimilation efficiency and initial length, but the number of individuals in the older cohorts is too large.

The cohort structure that results when respiration rate and assimilation efficiency are varied together is also unrealistic (Fig.

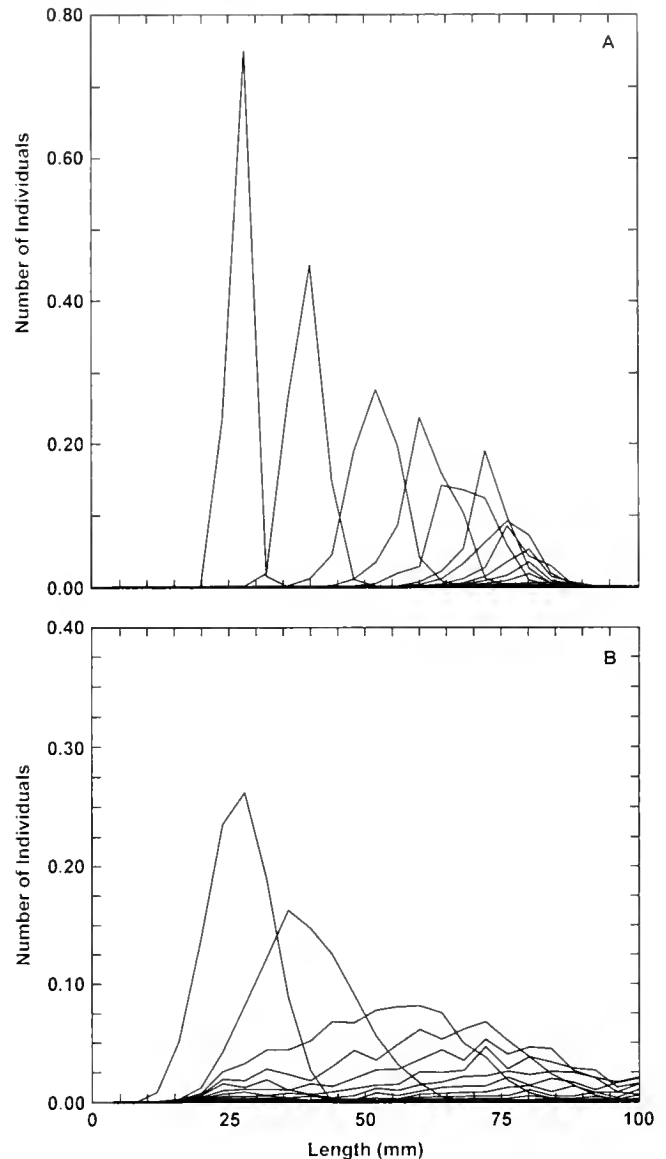


Figure 15. Simulated length-frequency distribution obtained for a hard clam cohort for (A) a reduced range of genetic variation imposed by setting  $\sigma_{C1}$  and  $\sigma_{C2}$  in Eq. (17) to 1.5 for initial length and 0.075 for assimilation efficiency, respectively, and (B) an expanded range of genetic variation imposed by setting  $\sigma_{C1}$  and  $\sigma_{C2}$  in Eq. (17) to 6.0 for initial length and 0.3 for assimilation efficiency.

16B) because the length range of 2-y-old clams at the beginning of the simulation is not adequately represented by a single initial length. The length effect disappears in later simulation years to produce cohort length distributions that are similar to those obtained by variations in assimilation efficiency and initial length (Fig. 14). Assimilation efficiency and respiration are in many ways equivalent physiological processes because each varies scope for growth. However, assimilation efficiency introduces a wider range of variability in cohort length structure and more accurately reproduces known cohort length structures, when only a single physiological characteristic is considered.

The interaction between clam initial length and variation in physiological rate is reflected in the distribution of clam lengths in the cohort at age 7 (simulation year 5). Low assimilation efficiency

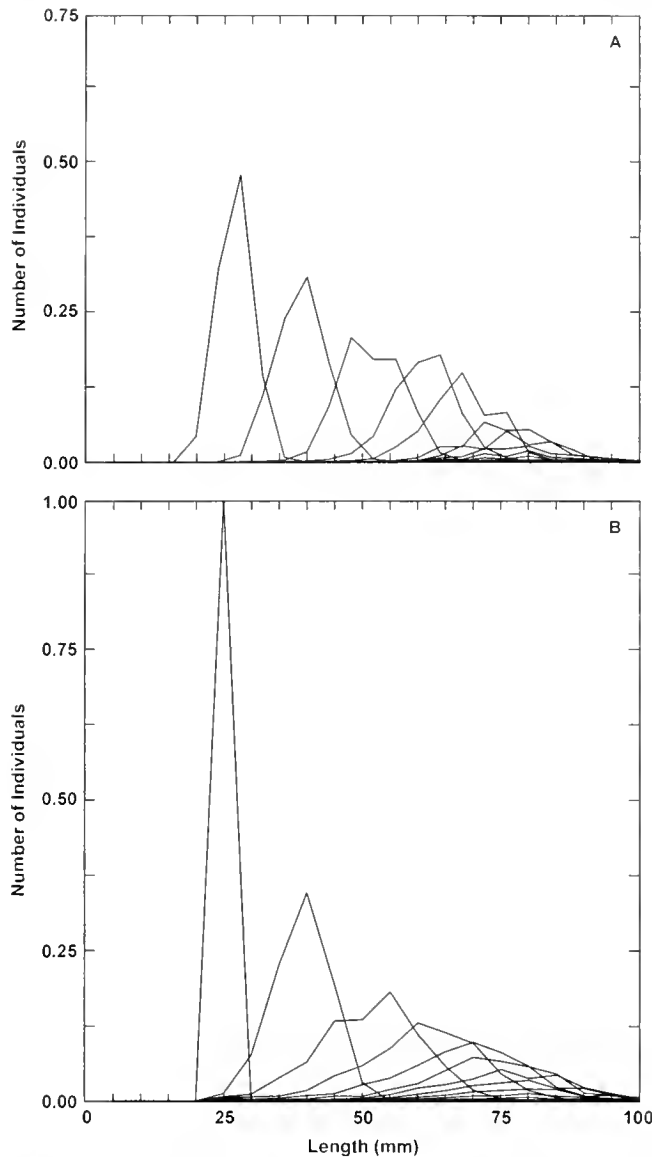


Figure 16. Simulated length-frequency distribution obtained for a hard clam cohort in which the population characteristics used to represent genetic variability were (A) initial clam length and respiration rate and (B) respiration rate and assimilation efficiency. The values of the genetic ranges are given in Table 2. The range of variation in respiration rate was assumed to be the same as for assimilation efficiency.

(less than 0.75 of the mean) results in hard clams that are <48 mm after 5 y, for all initial lengths (Fig. 17A). Production of clams larger than 85 mm requires assimilation efficiencies that are >1.25 times the mean rate, which gives a greater scope for growth. Variation of respiration rate over the same range gives larger clams for the same amount of change than does variation in assimilation efficiency (Fig. 17B). The implication is that larger clams are the more metabolically efficient, with lower weight-specific respiration rates. Changes in initial clam length for a given variation in respiration rate produce minor, but important modifications, to these trends.

The effect of variations in assimilation efficiency and initial length on total egg production is to constrain genotypes characterized by low assimilation efficiency and small initial size (Fig. 18A). Because more clams have average genotypes, the Gaussian-

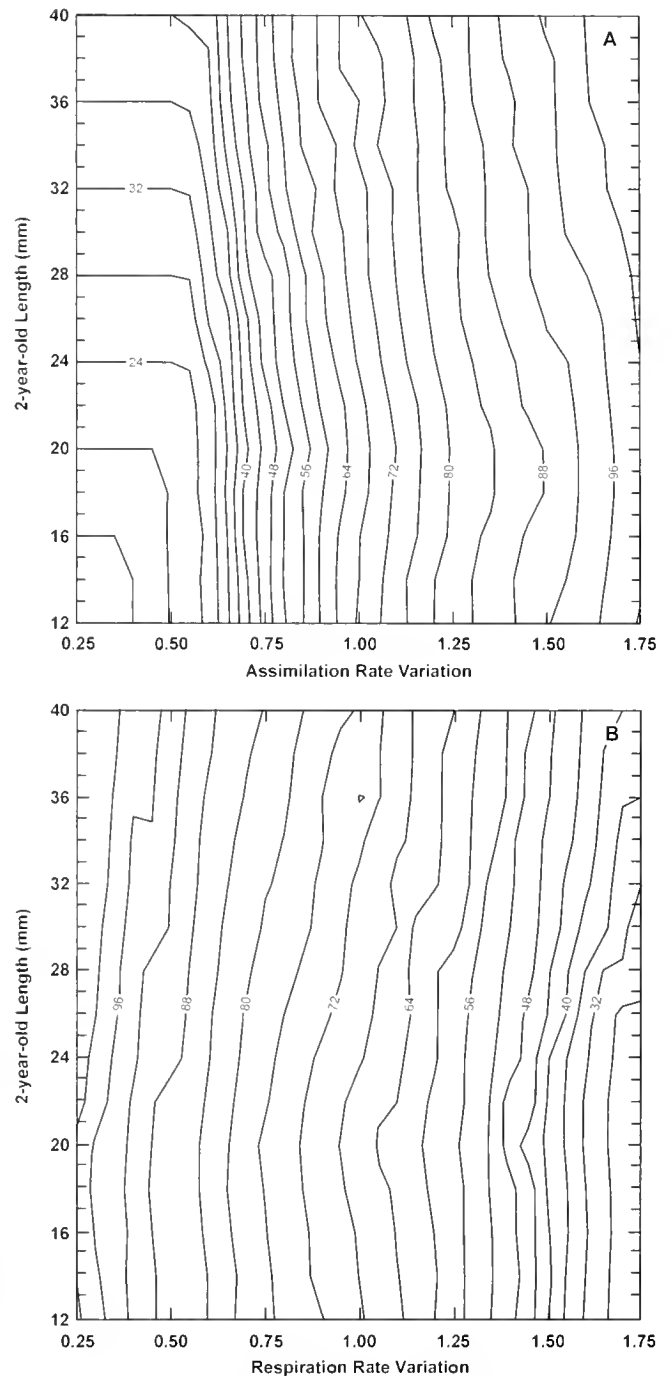


Figure 17. Simulated final length (mm) obtained for a hard clam cohort after five simulation years in which the population characteristics used to represent genetic variability were (A) initial clam length and assimilation efficiency and (B) initial clam length and respiration rate. The x-axis values represent the fraction of the mean value of all cohort genotypes as expressed by Eq. (17). The range in variation for respiration rate was assumed to be the same as for assimilation efficiency.

weighted egg production of the cohort will be highest near the center of the distribution, all else being equal. Assimilation rates (Fig. 18A) that are lower than the mean rate (<1) compress the Gaussian distribution, as these animals' scope for growth is insufficient to permit normal fecundity. Similarly, respiration rates that are higher than the mean rate (>1) (Fig. 18B) compress the Gaussian distribution, as high respiration debits energy that might oth-

erwise go into reproduction. The Gaussian-weighted egg production produced from the combination of respiration and assimilation efficiency rates (Fig. 18C) is slightly off center, indicating that the dominate members of the cohort responsible for reproduction are not the numerically most common genotypes. The most common genotype is at the center of the diagram (1.0, 1.0), whereas the center of egg production is below and to the right of this point (1.1, 0.95).

#### Hard Clam Population Simulations

##### Hard Clam Population Reference Simulation—Basic Characteristics

Concatenation of the yearly cohorts produced over a twenty-year simulation (Fig. 19A) results in a simulated hard clam population structure with two distinct abundance peaks centered around 25 mm and 40 mm and two smaller peaks at 60 mm and 70 mm. Highest abundance is associated with the newest cohort of 2-y-old clams at 25 mm modal size. The number of individuals at a given length (Fig. 19B) shows that most of the population is between 25 mm and 70 mm. For lengths greater than 70 mm, the number of individuals decreases as old-age mortality takes a toll.

The relationship between hard clam biomass and abundance (Fig. 19C) describes a trajectory that is followed as yearly cohorts are added to the population. The hard clam population initially peaks at about 2 g biomass and 0.8 individuals  $m^{-2}$  in year 4, after which it approaches a stable biomass of 6–7 g at an abundance of about 0.75 individuals  $m^{-2}$ . The population fluctuation that occurs as the first few cohorts are added is an artifact of model initialization and adjustment. The stability in the population after this adjustment represents a balance between the supportable biomass, the weight-dependent decline in fecundity in older clams, and the broodstock-recruitment relationship. Thus, the environmental conditions used in the reference simulation can support 6–7 g of hard clams, either as a few large or many small clams. The final simulated population abundance is typical of present day Great South Bay hard clam populations (Kraeuter et al. 2005).

The characteristics of the simulated population length frequency stem from an initial mode, centered around 25 mm (lower left, Fig. 20A), which moves towards larger lengths over time. This

mode is associated with the initial cohorts, which increase in length during the first few years of the simulation. After the initial adjustment years, the fraction of the population at a given length is relatively constant. A second mode appears after simulation y 4, again centered at 25 mm, which represents new recruits, which increase in length over time and are gradually absorbed into the larger length classes established by the initial cohorts. The hard clam population age-length structure after 20 y of simulation (Fig. 20B) shows that the fraction of the population at a given length spreads in age with increasing length and that the fraction of the population at a given age spreads in length as the population ages.

##### Hard Clam Population Reference Simulation—Sensitivity Studies

The simulated population structure is dependent on the form used for the broodstock-recruitment relationship (Eq. 30). Thus, the sensitivity of the population structure to different forms of this relationship was tested. The population structure that is produced by a broodstock-recruitment curve with no density-dependent control on population growth (Fig. 21A) shows more individuals  $m^{-2}$  and proportionately more smaller clams than obtained from the reference simulation (Fig. 19A). An equilibrium is not reached as each year sees an increase in the abundance of nearly all size classes (Fig. 21A). As a consequence, the population abundance at the end of the simulation is higher than observed for Great South Bay hard clam populations (Fig. 21B). The population age-length relationship is elongated towards larger animals (Fig. 22A) and curves upwards in age. The curvature indicates that large animals achieve higher abundance, which is consistent with no density-dependent controls. This is further indicated by the population trajectory (Fig. 22B), which shows an unconstrained increase in population biomass and number of individuals  $m^{-2}$  over the 20-y simulation.

Increasing the strength of the density-dependent control in the broodstock-recruitment relationship results in a simulated population structure in which the number of individuals  $m^{-2}$  (Fig. 23A) and the biomass (Fig. 23B) are reduced relative to the reference simulation (Fig. 19A, B). The relationship between population biomass and abundance (Fig. 23C) shows an initial adjustment, after which the population reaches a stable value of about 4 g at 0.4

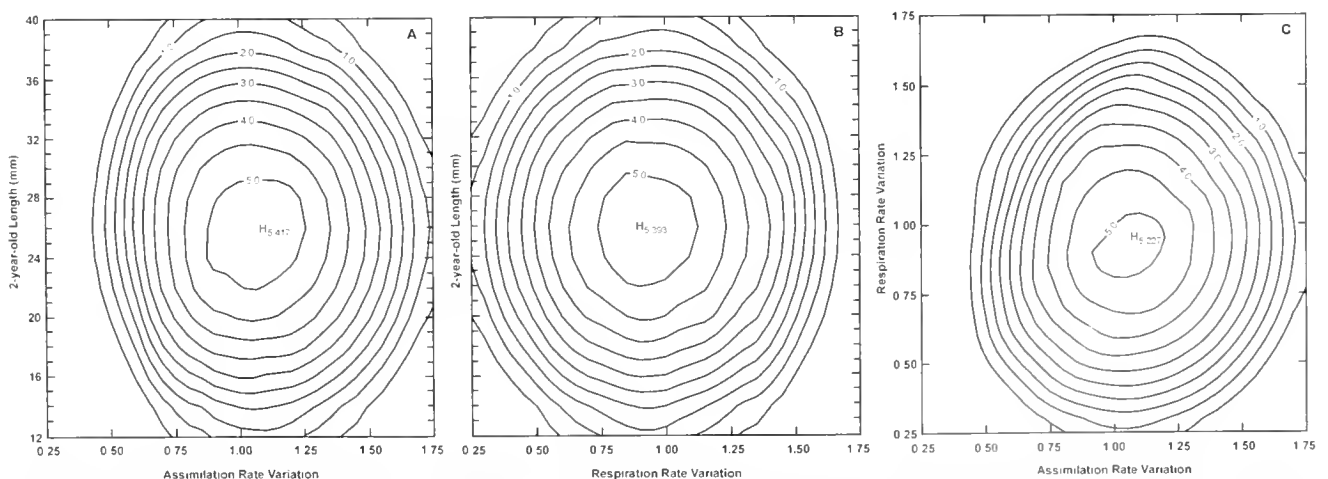


Figure 18. Egg production (contours are  $\log_{10}$  eggs produced) after five simulation years for a hard clam cohort with genetic variability introduced through variations in: (A) initial clam length and assimilation efficiency, (B) initial clam length and respiration rate and (C) respiration rate and assimilation efficiency. The x-axis values represent the fraction of the mean value of all cohort genotypes as expressed by Eq. (17). The range in variation for respiration rate was assumed to be the same as for assimilation efficiency. The number of eggs produced by a cohort is determined by the fecundity of the individual hard clam and the abundance of the genotype in the cohort.

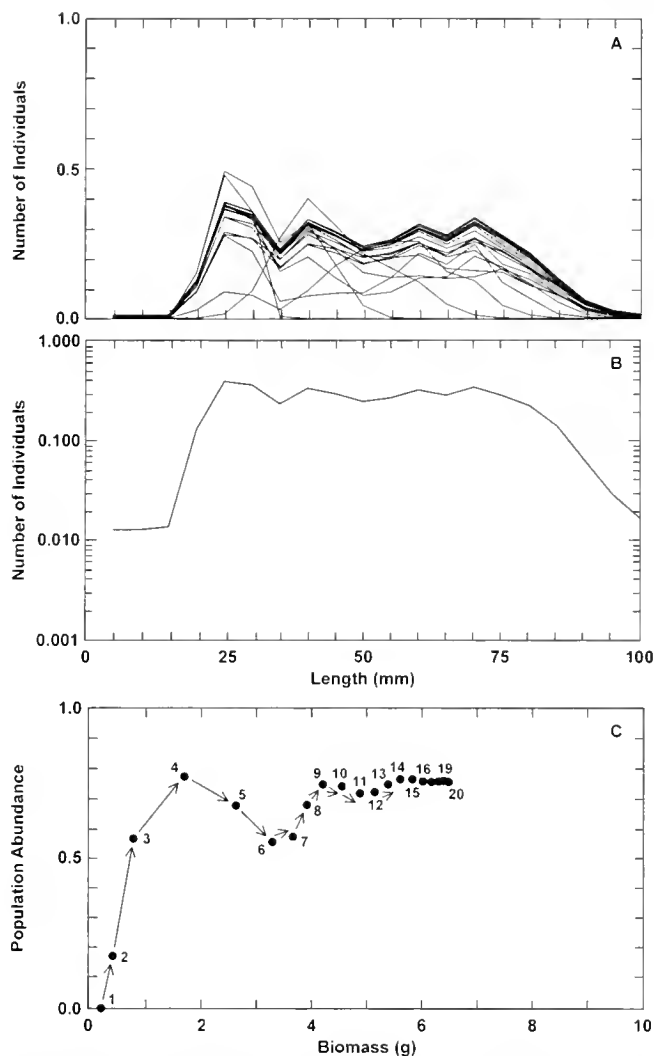


Figure 19. Simulated (A) population structure produced by summing a series of cohorts in which each line represents the length frequency of the population in a given year, (B) number of individuals at a given length in the population on a log scale and (C) the time history of population abundance as a function of biomass for hard clam populations exposed to the standard environmental conditions (Fig. 9) and for which genetic variability was represented by initial clam length and assimilation rate (Table 2). The superposed lines in the length-frequency distribution (A) identify the most stable age structure for the population.

ind  $m^{-2}$  and a stable length frequency (Fig. 23A). Abundance is too low, however, relative to observed hard clam population abundances in Great South Bay.

Natural mortality rate also affects the simulated population structure. Increasing the mortality rate for 40-to-60-mm hard clams produces a stable population structure (Fig. 24A), but the number of individuals  $m^{-2}$  at a given length is about an order of magnitude less than that obtained for the reference simulation (Fig. 24B versus Fig. 19B). The population abundance as a function of population biomass stabilizes at a value of about 0.05 g and 0.15 ind  $m^{-2}$  (Fig. 24C), values much below those observed for Great South Bay hard clam populations. Removal of the intermediate-size clams at an increased rate skews the population age-length relationship (Fig. 25) towards smaller lengths and younger ages by limiting the number of animals that grow to large size.

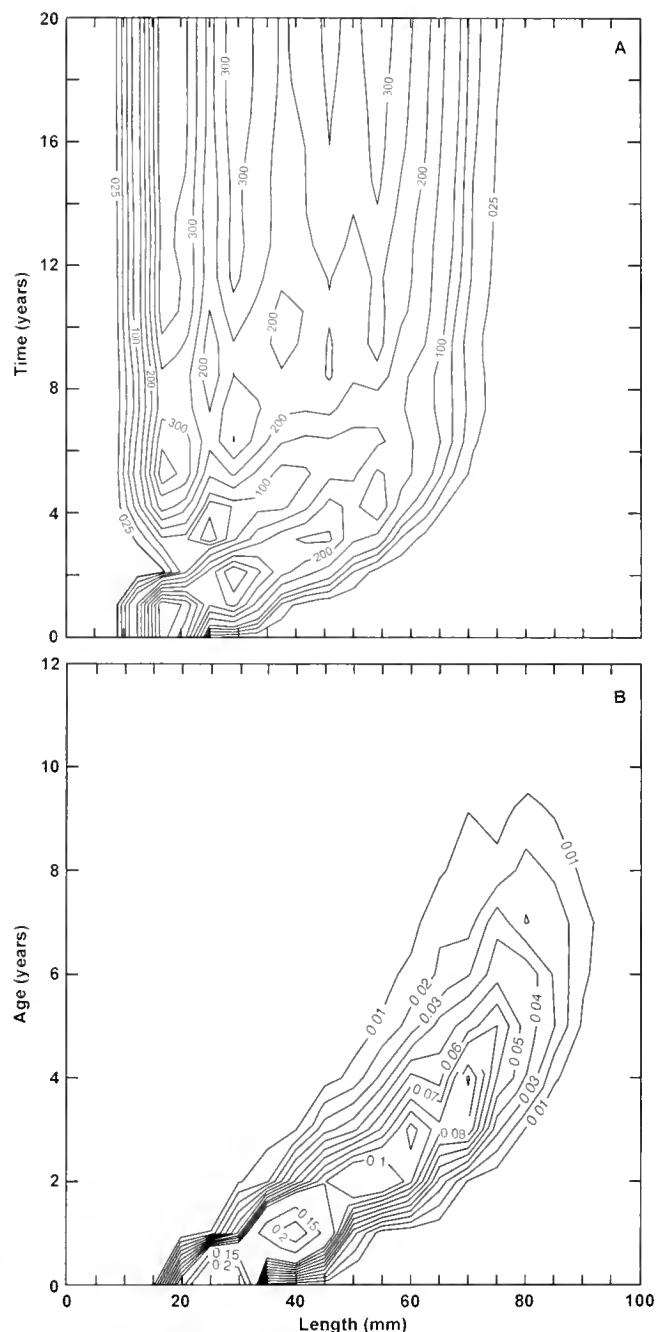


Figure 20. Simulated change in hard clam population length as a function of (A) simulation time, showing the maturity of the population length structure and (B) hard clam age, showing the age-length structure of the mature population after 20 y of simulation time. Contours are number of individuals  $m^{-2}$ .

Increased mortality of 60-to-80-mm hard clams results in a population that stabilizes at a biomass and abundance (Fig. 26A) of about 1.3 g and 0.36 ind  $m^{-2}$ , respectively, still well below Great South Bay observations, but higher than the 40–60 mm simulation (Fig. 24) because fecundity per g dry wt is highest in clams of this latter size range and so recruitment is increased. The age-length structure of the population is truncated at lengths above 60–70 mm and ages in excess of simulation years 4 and 5, which correspond to clam age years of 6 and 7 (Fig. 26B).

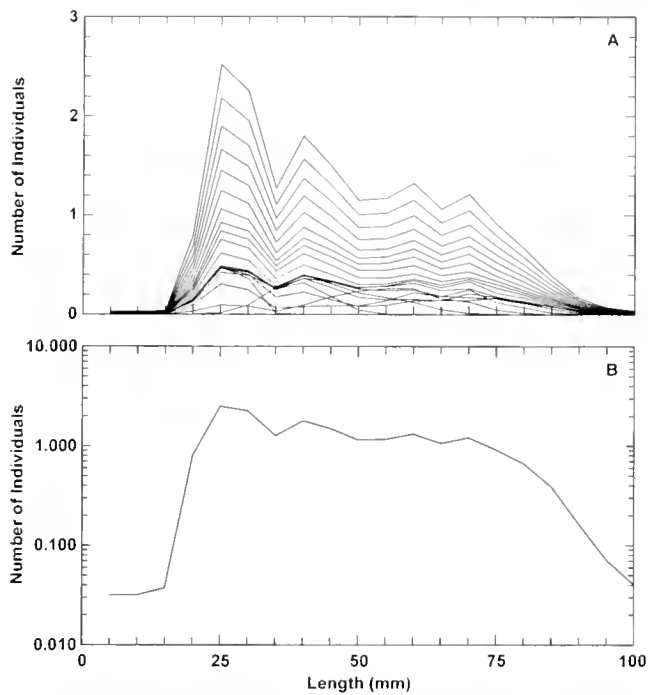


Figure 21. Simulated (A) population length frequency in which each line represents a yearly state of the population and (B) the number of individuals at a given length obtained for a hard clam population in which the density-dependent constraint in the broodstock-recruitment relationship was removed.

#### Simulated Age-length Relationships

The age-length distribution obtained from the reference simulation (Fig. 27A) shows an almost linear relationship between length and age for hard clams between 20 and 40 mm, after which the increase in length for an incremental increase in age slows, giving a curvature to the distribution. The upward curvature of the age-length distribution is produced by reduction in growth rate with increasing length, which is consistent with observations (e.g., Ansell 1968, Loesch & Haven 1973, Devillers et al. 1998). Older hard clams do not increase in length as rapidly as they age. The newest two cohorts (2- and 3-y-old animals, simulation ages of 0 and 1 y) establish discrete modes in the population length- and age-frequency distribution; older cohorts meld into a single mode characterized by decreasing numbers as the older cohorts age (Fig. 27A).

The distribution of lengths obtained from sections across the age-length relationship at given ages produces a typical length-frequency diagram that becomes increasingly skewed with increasing cohort age (Fig. 27B). The tail of the distribution extends towards smaller length classes because some genotypes grow slowly and hence continually fall behind the cohort modal length. The differential growth rates of the individuals in a cohort produces, in part, the asymmetric distribution in age-length space (Fig. 27A) that is skewed towards smaller lengths and older ages. Sections through the hard clam age-length distribution at specific lengths shows that the age distribution becomes increasingly skewed at increasingly larger lengths (Fig. 27C). The long tail that extends towards older age classes occurs because the rate of natural mortality increases with clam age. The assumption that a cohort (or length class) has a Gaussian distribution of lengths (ages) is

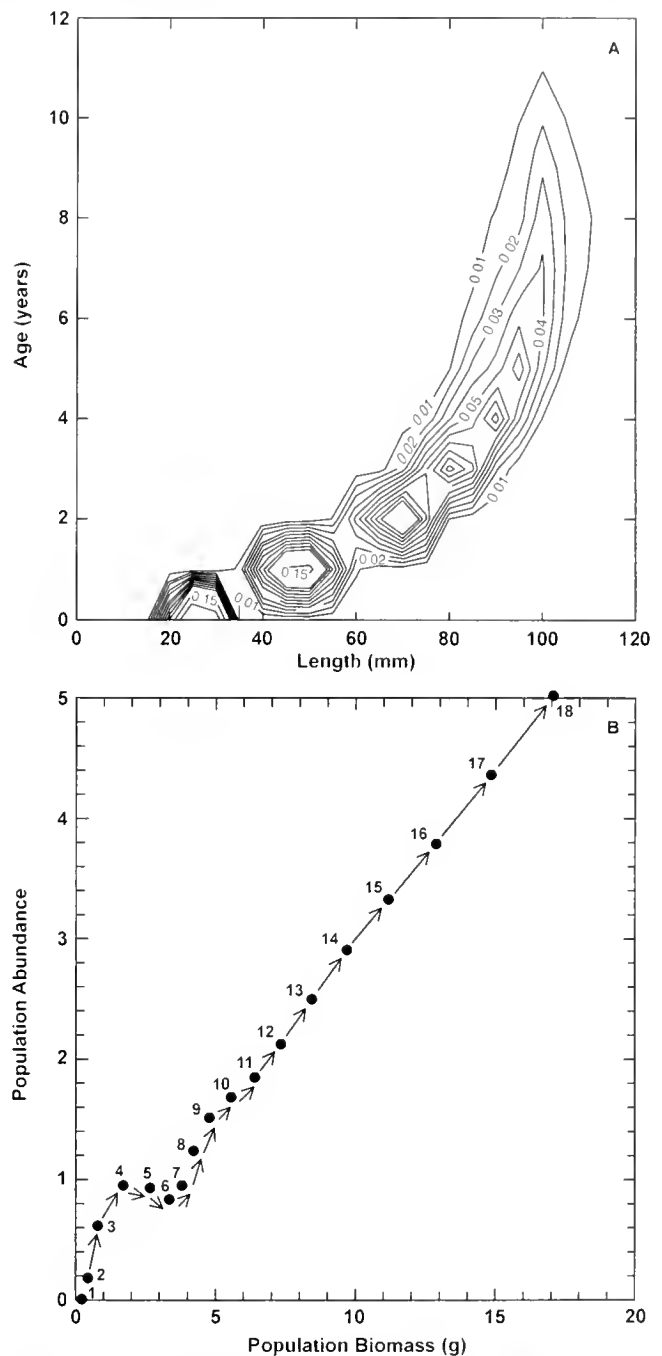


Figure 22. Simulated (A) age-length relationship and (B) the time history of population abundance as a function of biomass obtained for a hard clam population in which the density-dependence constraint in the broodstock-recruitment relationship was removed. Contours are number of individuals  $m^{-2}$ .

correct for smaller ages (lengths) (Craig & Oertel 1966), but the Gaussian approximation becomes increasingly less accurate at older ages and larger lengths.

The hard clam model provides a framework for ascribing causes to shape variations in age-length distributions. Variations in cohort growth rates expand (Fig. 28A) or contract (not shown) the population expression in age-length space along the long axis. Faster growth tends to maintain the Gaussian shape of the age-

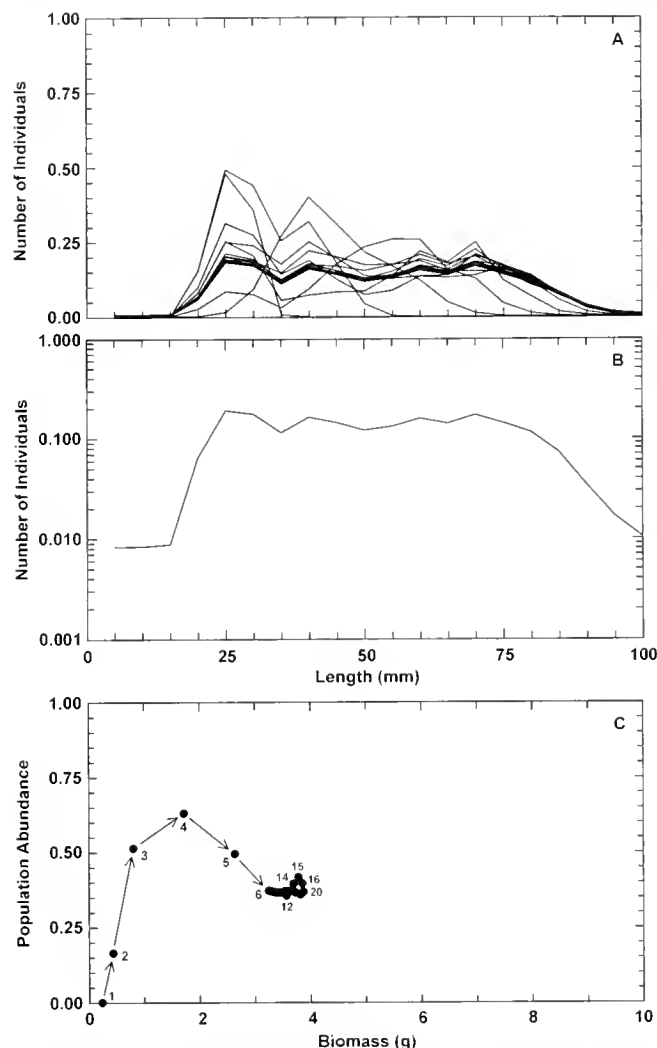


Figure 23. Simulated (A) population length frequency in which each line represents a yearly state of the population, (B) the number of individuals at a given length and (C) the time history of population abundance as a function of biomass for a hard clam population in which the density-dependence constraint in the broodstock-recruitment relationship was increased.

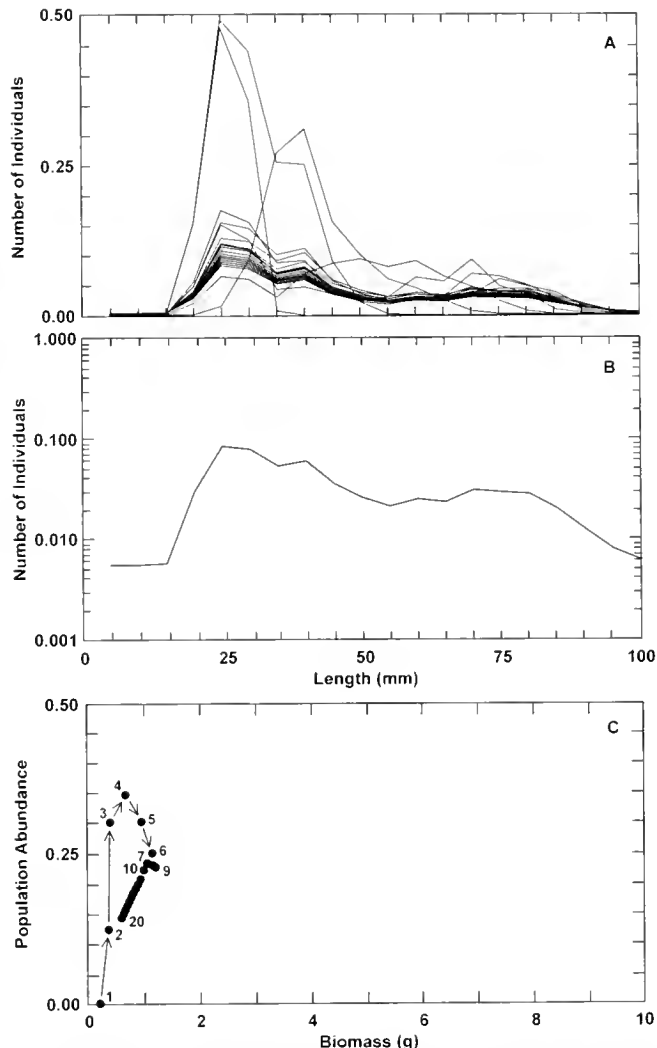


Figure 24. Simulated (A) population length frequency in which each line represents a yearly state of the population, (B) the number of individuals at a given length and (C) the time history of population abundance as a function of biomass for a hard clam population in which mortality imposed on the 40- to 60-mm clams was increased.

frequency and length-frequency distributions (Figs. 28B, C); whereas, slow growth tends to exaggerate the asymmetry in both directions.

Decreased adult mortality rate yields an age-length distribution (Fig. 29A) that is similar to that obtained for the reference simulation (Fig. 27A). However, the asymmetry in the age-frequency distribution is greater because of the tendency for age-dependent mortality to be biased towards older ages regardless of length. A decrease in mortality rate has only a minor influence on the length frequency for a given cohort (Fig. 30A), because mortality is apportioned by cohort; that is, mortality is age-dependent rather than length dependent. In contrast, the age frequency for a given length (Fig. 30C) varies from the reference simulation. In this case, a larger fraction of animals in the larger length classes (e.g., 70 mm) are represented by the oldest clams.

Increased adult mortality rate results in an age-length distribution that is compressed in age for a given length (Fig. 29B). However, the length frequency at age is similar to that from the reference simulation (Fig. 27B) and the decreased adult mortality simu-

lation (Fig. 30A) because relatively more large animals are old, which truncates the length frequency at large size. The age frequency at length (Fig. 30D) is less skewed than in the reference simulation (Fig. 27C) because the older animals that add skewness to the distribution are present in much lower numbers.

Reducing recruitment by half in the last five years of the simulation narrows the age-length distribution at small length (Fig. 31A). In this simulation, a smaller fraction of the population is represented over a range of age-at-length (Fig. 31B) and length-at-age (Fig. 31C) distributions. Relative to the reference simulation (Fig. 27B), the length frequencies of older clams are unchanged, whereas the length frequency of the 2-y-old cohort is muted by reduced recruitment. In contrast, all age frequencies are muted in comparison with the reference simulation (Fig. 27C) because some animals at nearly all lengths were  $\leq 5$  y old. Hence, the distribution of age-at-length has been affected more than the distribution of length-at-age.

The shape of the hard clam population age-length distribution is also controlled by the cohort genetic composition. A restricted



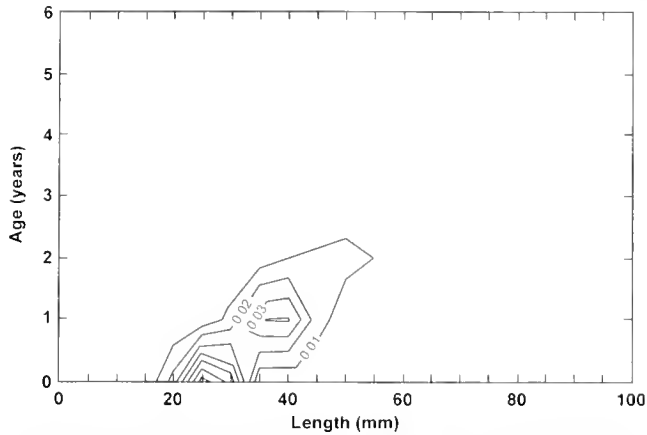


Figure 25. Simulated age-length relationship for a hard clam population in which mortality imposed on the 40-to-60-mm clams was increased. Contours are number of individuals  $m^{-2}$ .

proportion of slow and fast growers in the cohort, which reflects numerical abundance of a narrow range of genotypes, yields a markedly different population structure (Fig. 32A). This age-length composition is similar to that obtained with increased

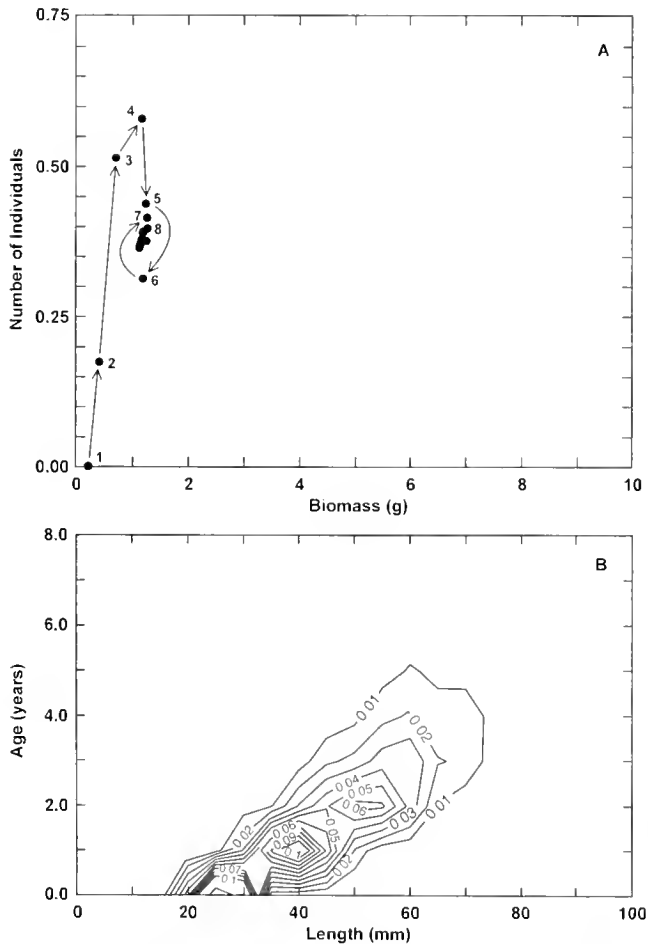


Figure 26. Simulated (A) time history of population abundance as a function of biomass and (B) age-length relationship for a hard clam population in which mortality imposed on the 60-to-80-mm clams was increased.

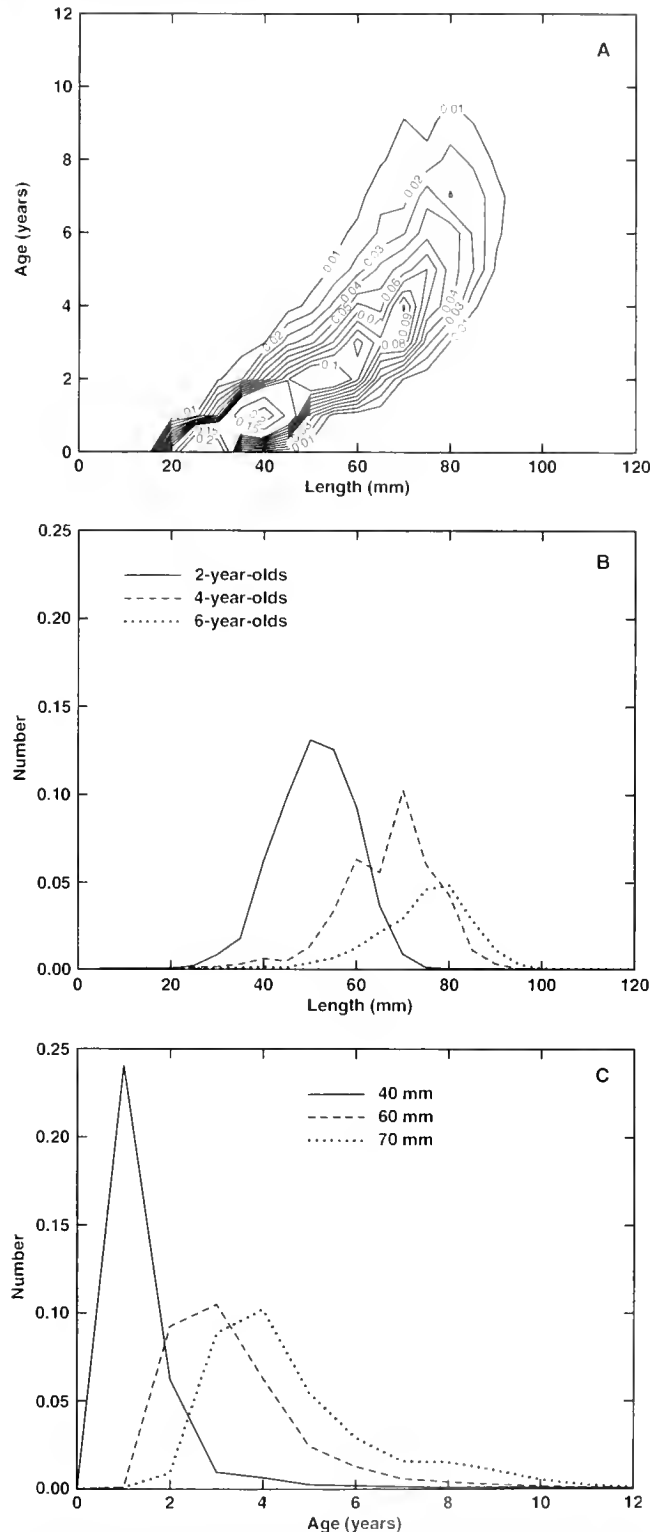


Figure 27. Simulated (A) age-length (B) length-at-age and (C) age-at-length frequency distributions obtained from the reference simulation that used standard environmental conditions for Great South Bay, NY. Contours are number of individuals  $m^{-2}$ .

growth rate (Fig. 28). The degree of bending along the long axis is increased with respect to the reference simulation (Fig. 27A) because more individuals in the cohort have the average genotype and this also constrains the width of the age-length distribution in

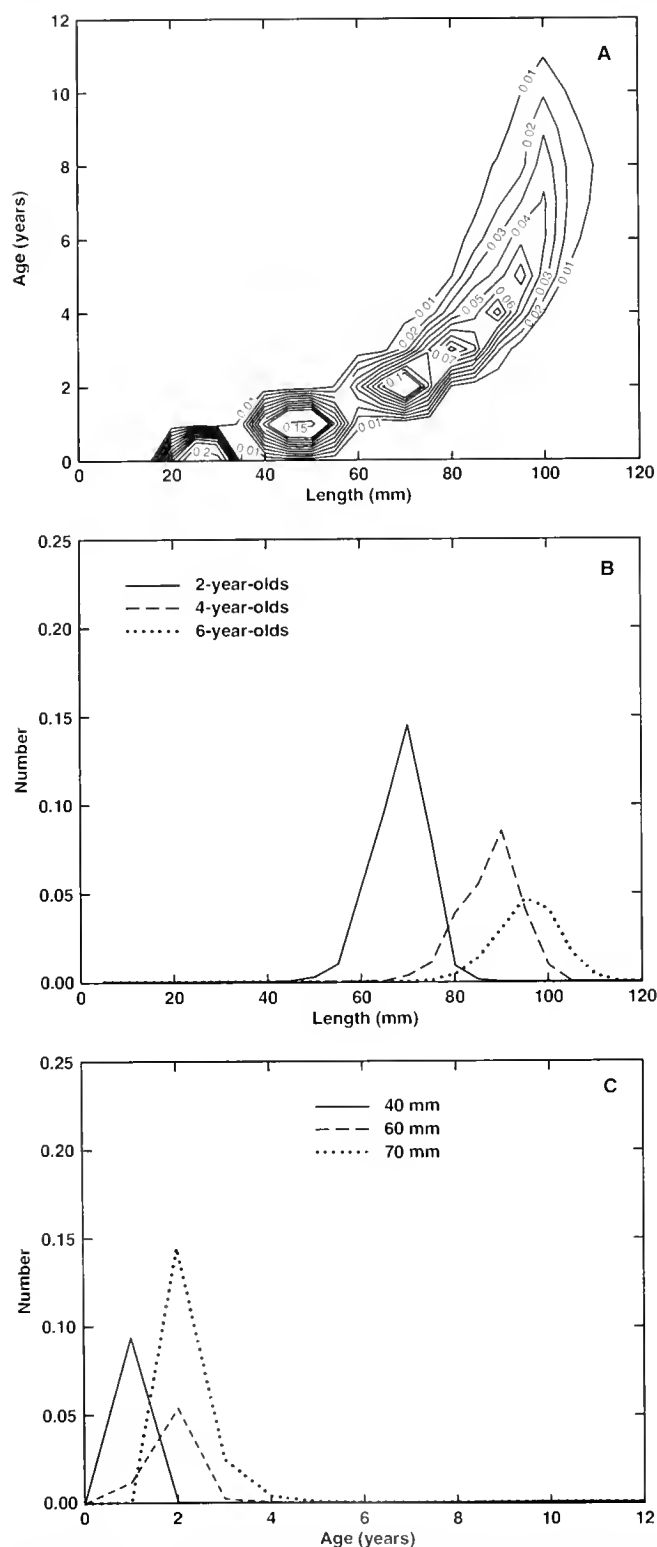


Figure 28. Simulated (A) age-length (B) length-at-age and (C) age-at-length frequency distributions obtained when the population growth rate is increased. Contours are number of individuals  $m^{-2}$ .

age-length space. The range of lengths at age is small, as is the range of ages at length. Because the increase in length ceases relatively uniformly within the cohort, the increment in age for a given increment in length increases with age, producing a strongly curved age-length distribution.

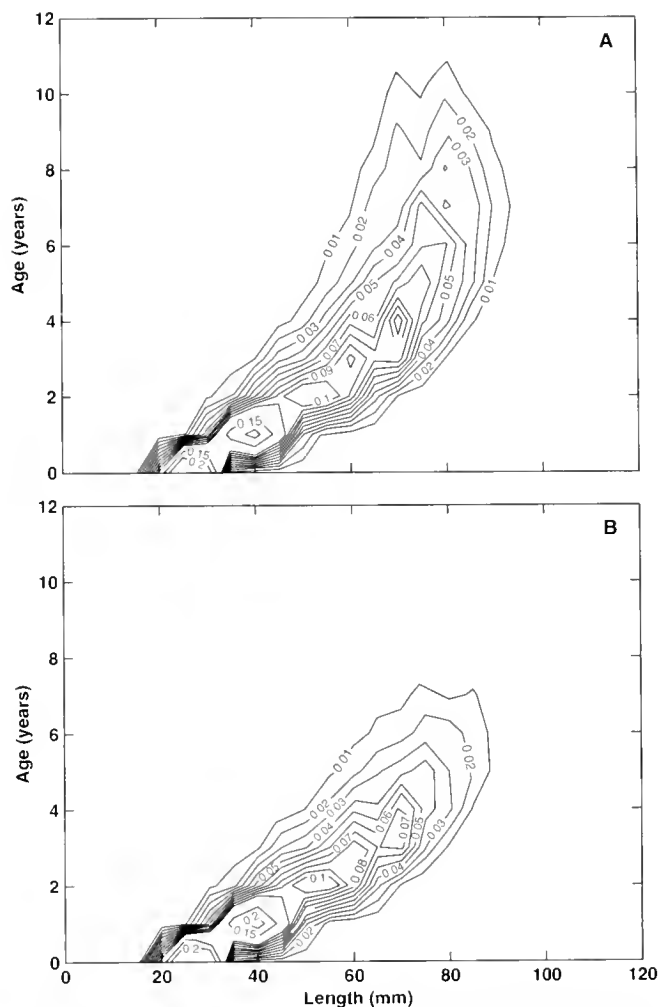


Figure 29. Simulated age-length distributions obtained when adult mortality rate is A) decreased and B) increased. Contours are number of individuals  $m^{-2}$ .

An expanded proportion of slow and fast growers in the cohort, which provides more even representation of genotypes, substantially changes the appearance of the hard clam population (Fig. 32B). The degree of bending along the long axis is lessened because more individuals in the cohort grow faster at older age. However, more individuals in the cohort also grow slower at older ages. As a consequence, the age-length distribution expands along both axes. The range of lengths at age is wider, as is the range of ages at length. These two trends straighten the shape of the population structure in age-length space, but also truncate the length of the long axis because fewer very-old and very-large animals exist.

#### Development of General Age-length Relationship

The age-length distributions obtained from the simulated populations (Figs. 27, 28, 30, 31, 32) have common characteristics. Each is inclined at an angle to the age-length axes, the long axis typically twists upwards, and the asymmetry along the long axis typically increases with progression towards older, larger clams. The asymmetry in these age-length distributions is internally consistent in that a more restricted length and age range of clams falls on the younger and larger side of the long-axis ridge. The curvature in the location of the ridge as the clams get older indicates that a maximum length exists for the population and that older clams

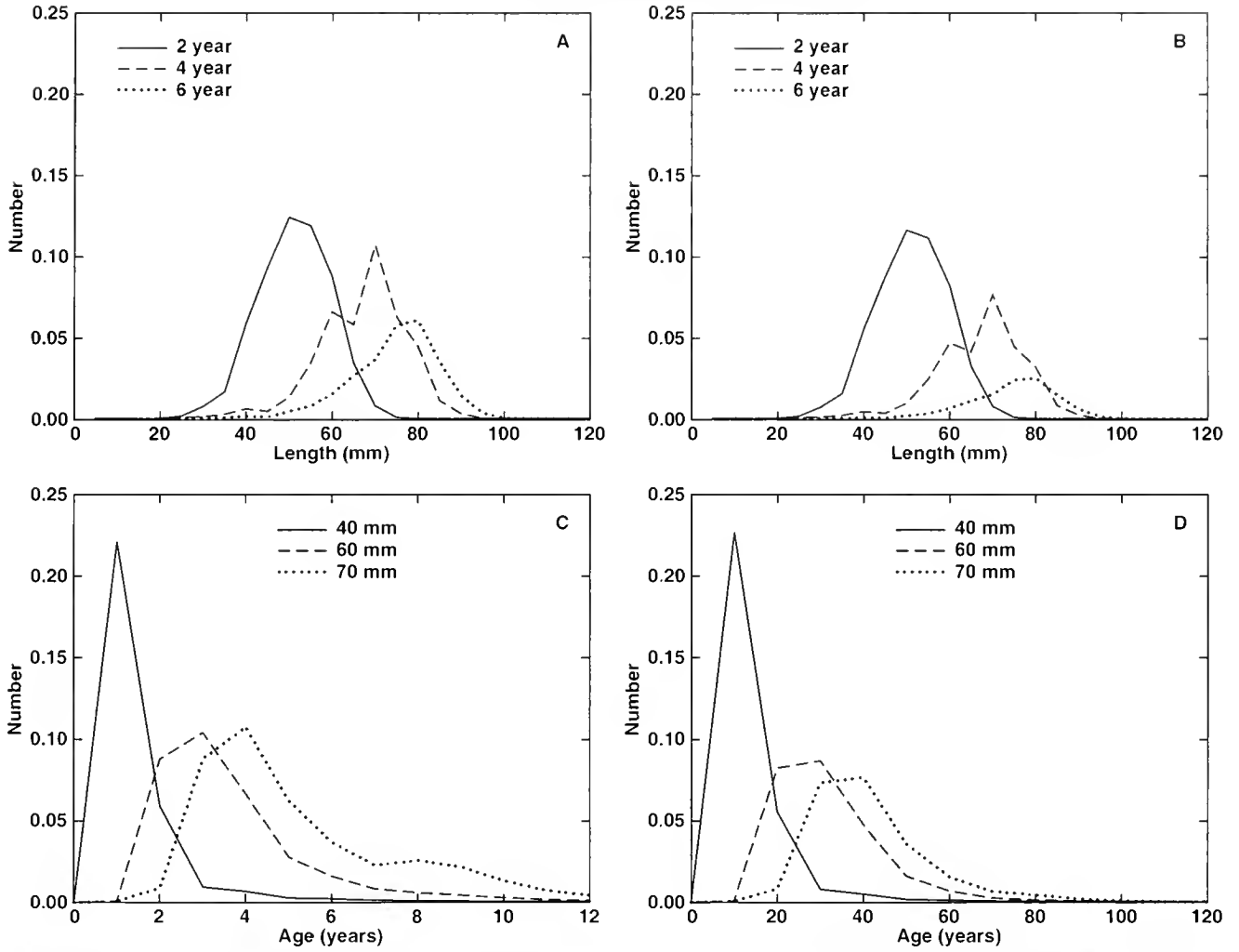


Figure 30. Simulated age-at-length obtained for (A) decreased and (B) increased adult mortality rate and simulated length-at-age obtained for (C) decreased and (D) increased adult mortality.

live for a while near their largest length. The age-at-length and length-at-age slices through the various age-length relationships show that these follow an approximate Gaussian distribution (e.g., Fig. 30). The similarity in the form of the various age-length distributions obtained from simulations that included a range of different processes suggests that they can be described by a general mathematical relationship, as developed in the following sections.

#### Formulation of a Twisted Bivariate Gaussian Functional Form

The structure of the age-length relationship can be reproduced by a bivariate Gaussian function that includes the capabilities for curvature at increasing age and length and for variability in the distribution about a given age or length. The bivariate Gaussian function was implemented in a rotated coordinate system ( $x, y$ ) that is aligned along the axis of the maximum in the length-age ( $L, A$ ) distribution in the original coordinate system (Fig. 33). The alignment is done using a coordinate rotation angle  $\theta$ , which shifts the new coordinate system counterclockwise relative to the age axis. The rotated coordinate system is centered around a central age ( $A_0$ ) and length ( $L_0$ ) that are representative values associated with the maximum in the age-length distribution. These parameters control the location of the origin of the rotated coordinate system relative

to the original age-length distribution. The  $x$  and  $y$  axes in the rotated coordinate system are scaled by the factors  $S_A$  and  $S_L$ , which allows matching of the magnitude of the age and length values in the two coordinate systems. The coordinate transformation is:

$$\begin{pmatrix} x \\ y \end{pmatrix} = \begin{pmatrix} \cos \theta & \sin \theta \\ -\sin \theta & \cos \theta \end{pmatrix} \cdot \begin{pmatrix} S_L(L - L_0) \\ S_A(A - A_0) \end{pmatrix} \quad (31)$$

and the correspondence between the original age-length coordinate system and the rotated coordinate system is shown in Figure 33.

The transformed coordinate system aligns the age-length distribution so that it can be fit with a bivariate Gaussian function to obtain the number of individuals  $m^{-2}$  at a given length and age,  $N(x, y)$ , as:

$$N(x, y) = N_0 e^{\frac{-x^2}{2\sigma_L^2}} e^{\frac{-(x-y)m^2}{2\sigma_A^2}} \quad (32)$$

where  $N_0$  is the maximum number of individuals  $m^{-2}$  at zero age,  $x$  and  $y$  are the transformed length and age axes, respectively, and  $\sigma_L$  and  $\sigma_A$  determine the spread of the Gaussian, the standard deviation, along the length and age distribution, respectively. The

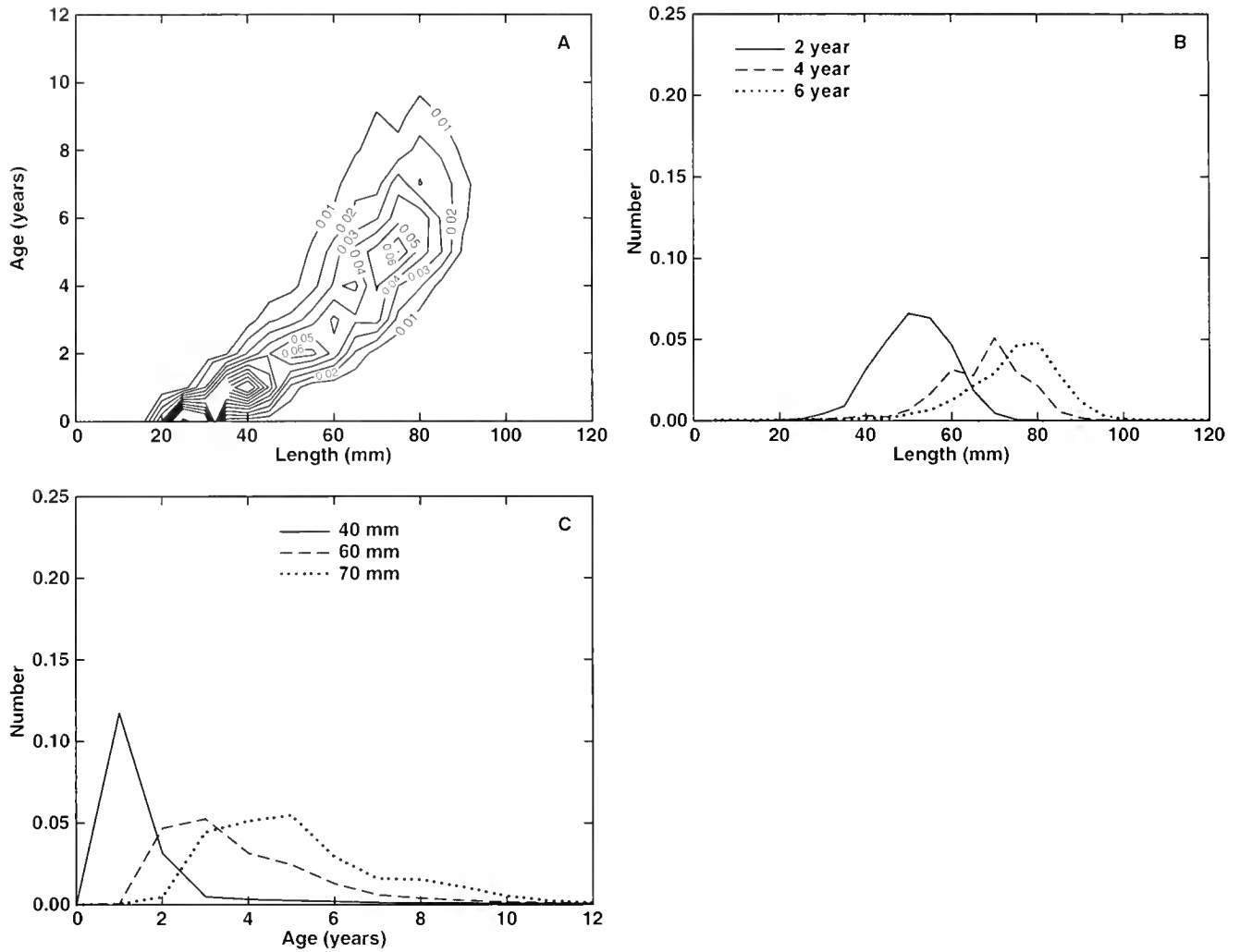


Figure 31. Simulated (A) age-length (B) length-at-age and (C) age-at-length frequency distributions obtained when the recruitment rate is decreased. Contours are number of individuals  $\text{m}^{-2}$ .

parameter  $y_m$  is the center of the Gaussian distribution along the rotated age axis ( $y$ ).

The typical age-length distribution (e.g., Fig. 27A) has three additional features that need to be included in Eq. (32). First, the ridge in age-length space curves upward for older and longer clams. The curvature of the age-length distribution at older and longer clams is introduced by allowing the center of the Gaussian distribution, given by  $y_m$ , to change as a function of the rotated  $x$  coordinate:

$$y_m = a x^n \quad (33)$$

which shifts the centroid of the Gaussian towards positive  $y$  with increasing  $x$ , with the degree of curvature determined by  $n$  (Fig. 33). This modification to  $y_m$  provides the twist to the bivariate Gaussian.

Second, the age-length distribution has a shallower slope (is wider) for older (longer) clams relative to younger (shorter) clams, which produces an asymmetric distribution. The asymmetry in the spreading of the age-length distribution about the maximum ridge value is created by allowing  $\sigma_y$  to vary along the rotated age axis ( $y$ ).

Third, the width of the age-length relationship increases for older and longer clams. The spreading of the age-length distribu-

tion for older and longer clams is created by increasing the value of  $\sigma_y$  with distance along the rotated length axis,  $x$ , as:

$$\sigma_y(x, y) = \begin{cases} S1(1 + b1x), & \text{if } y - y_m > 0, \\ S2(1 + b2x), & \text{if } y - y_m < 0 \end{cases} \quad (34)$$

where the parameters  $S1$ ,  $S2$ ,  $b1$  and  $b2$  are obtained from fitting the general age-length relationship to an age-length data set, as described in the following section. This modification allows  $\sigma_y(x, y)$  to have different values on either side of the maximum ridge value,  $y_m$ .

The addition of the above modifications to Eq. (32) gives a twisted bivariate Gaussian of the form:

$$N(x, y) = N_0 e^{\frac{-x^2}{2\sigma_x^2}} e^{\frac{-(y - y_m(x))^2}{2\sigma_y(x, y)^2}} \quad (35)$$

The application of Eq. (35) to determine age-length distributions is described in the following section.

#### Functional Fitting Procedure

The twisted bivariate Gaussian function (Eq. 35) has 13 parameters that need to be specified to create an age-length distribution. The coordinate scaling factors ( $S_L$ ,  $S_A$ ) and the Gaussian width in the same direction ( $\sigma_x$ ,  $\sigma_y$ ) are not independent param-

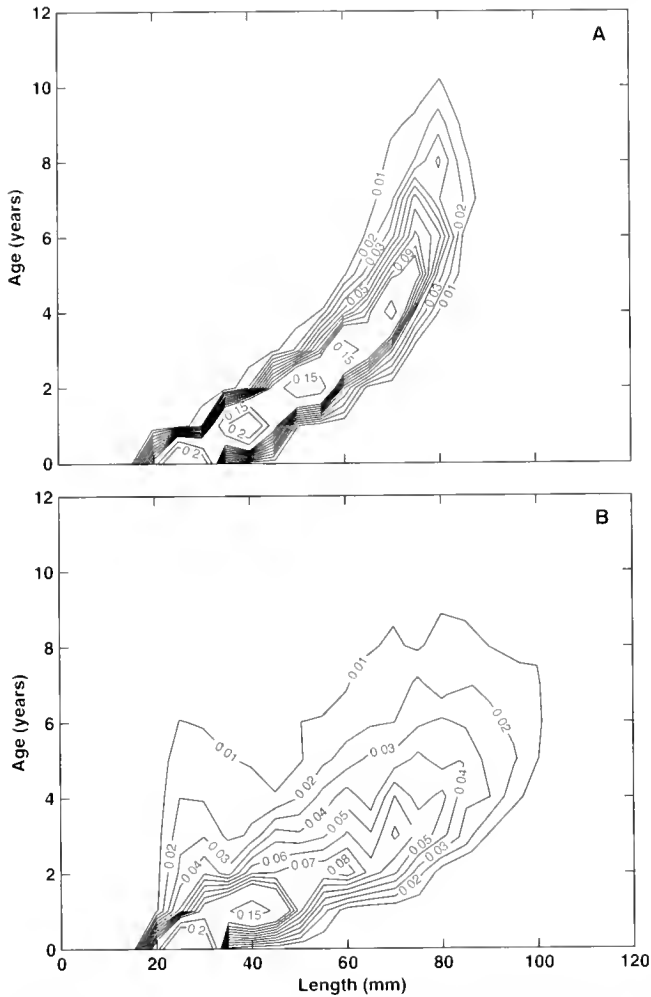


Figure 32. Simulated age-length frequency distributions obtained when the range of assimilation efficiency genotypes was (A) narrower and (B) broader in comparison with the reference simulations. Contours are number of individuals  $\text{m}^{-2}$ .

eters, so the values of  $S_L$  and  $S_A$  were specified and the Gaussian width parameters were determined. The rotated  $(x, y)$  coordinate system is constrained to be on the  $A = 0$  axis by choosing  $A_0 = 0$ . The remaining 10 parameters (Table 4) need to be determined.

The parameter fitting procedure is based on minimizing the difference in hard clam age-length distributions obtained from observations and those obtained from the twisted bivariate Gaussian model (Eq. 35). Prior to the start of this procedure, the age-length data sets were filtered relative to a threshold value because many age-length combinations do not occur in hard clam populations. These combinations were removed from the input data sets by setting a minimum length criterion for each age-length combination. For this study, the maximum clam density in the simulated age-length distributions, which is controlled by the total number of clams in the population model, is 0.25 clams of a given age and length  $\text{m}^{-2}$ . The threshold value for the age-length distributions was set at 6% of this maximum value, 0.015 clams  $\text{m}^{-2}$ .

The parameter fitting procedure is based on minimizing the sum square deviation between the observed number of clams  $\text{m}^{-2}$  ( $N'_i$ ) and the theoretically-derived number of clams  $\text{m}^{-2}$  ( $N''_i$ , obtained from Eq. 35) for a given length ( $L_i$ ) and age ( $A_i$ ) as:

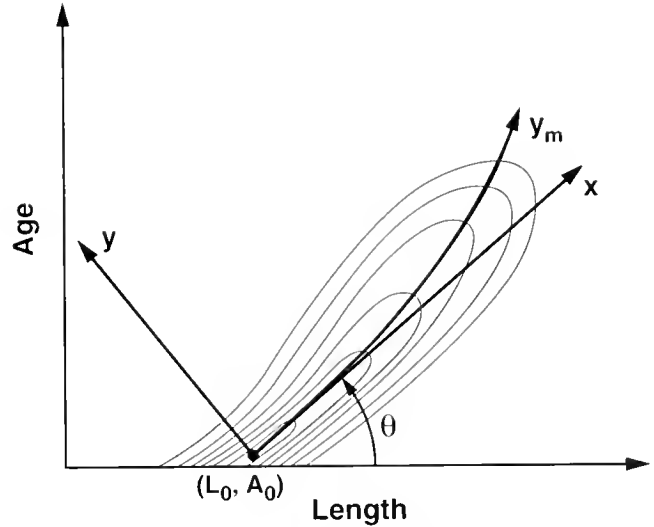


Figure 33. Schematic of correspondence between the age-length coordinate system and the scaled age-length coordinate system used for the twisted bivariate Gaussian function. The axes  $(x, y)$  of the scaled coordinate system have an origin at  $(L_0, A_0)$  and are rotated relative to the original length and age axes by the angle  $\theta$ . The curvature of the age-length distribution is given by  $y_m$ . See text for additional details of the scaled age-length coordinate system.

$$C = \sum_i (N''_i - N'_i)^2. \quad (36)$$

where  $C$  is a measure of the misfit between the observed and theoretical age-length distributions.

The minimization procedure is most efficient when started with initial parameter values that are near optimal values. The initial parameter values were estimated by an iterative procedure in which the minimum and maximum values for a particular parameter were used to set a range for the parameter. Values at 25%, 50% (midpoint) and 75% of this range were then determined and these along with the two extremes were used in Eq. (35) and the misfit between the theoretical and observed age-length distribution was determined with Eq. (36). The parameter set that produced the minimum misfit was taken as the initial parameter values and the minimization procedure repeated to yield a second estimate of the optimal parameter set. This parameter set was then used as initial values and the minimization procedure repeated a third time. The parameter values that were obtained from this minimization provide the starting point for a multidimensional minimization procedure, based on Powell's Method (Press et al. 1989), which produces the final optimal parameter set. This final parameter set provides the best fit of the twisted bivariate Gaussian model to a particular age-length data set. Once the optimal parameter set is determined, the twisted bivariate Gaussian model can be used to develop an age-length distribution that includes all ages and lengths.

#### Parameterization of Simulated Age-length Relationships

The age-length data sets obtained from the simulations in which growth, mortality and recruitment rates were varied, and in which the apportionment of individuals among genotypes was expanded and reduced, were used to fit the 10 free parameters for the twisted bivariate Gaussian function (Table 4). The resulting parameters were then used with Eq. (35) to produce age-length distributions (Fig. 34).

TABLE 4.

Optimal parameter values obtained from fitting the twisted bivariate Gaussian given by eq. (35) to different simulated hard clam population age-length data sets. The figure showing the corresponding age-length distributions is indicated.

	Reference Case	Decreased Growth	Increased Growth	Increased Adult Mortality	Decreased Adult Mortality	Decreased Recruitment	Reduced Genetics	Expanded Genetics
$L_0$	27.5	25.2	29.9	25.0	26.9	28.2	27.6	21.8
$\theta$	0.550	0.879	0.260	0.553	0.490	0.551	0.414	0.258
$a$	0.0333	-0.0055	0.0074	0.0198	0.0501	0.0361	0.0778	0.0401
$n$	2	2	3	2	2	2	2	2
$S_1$	0.322	0.312	0.366	0.197	0.361	0.390	0.352	0.637
$b_1$	0.158	0.155	0.111	0.158	0.158	0.158	0.044	0.355
$S_2$	0.235	0.479	0.519	0.322	0.322	0.176	0.152	0.511
$b_2$	0.080	0.022	-0.155	0.041	0.100	0.080	0.102	-0.199
$N_0$	0.183	0.078	0.172	0.201	0.151	0.083	0.283	0.178
$\sigma_1$	4.46	3.36	5.17	3.83	5.09	6.00	4.18	4.00
Figure No.	27A	not shown	28A	29B	29A	31A	32A	32B

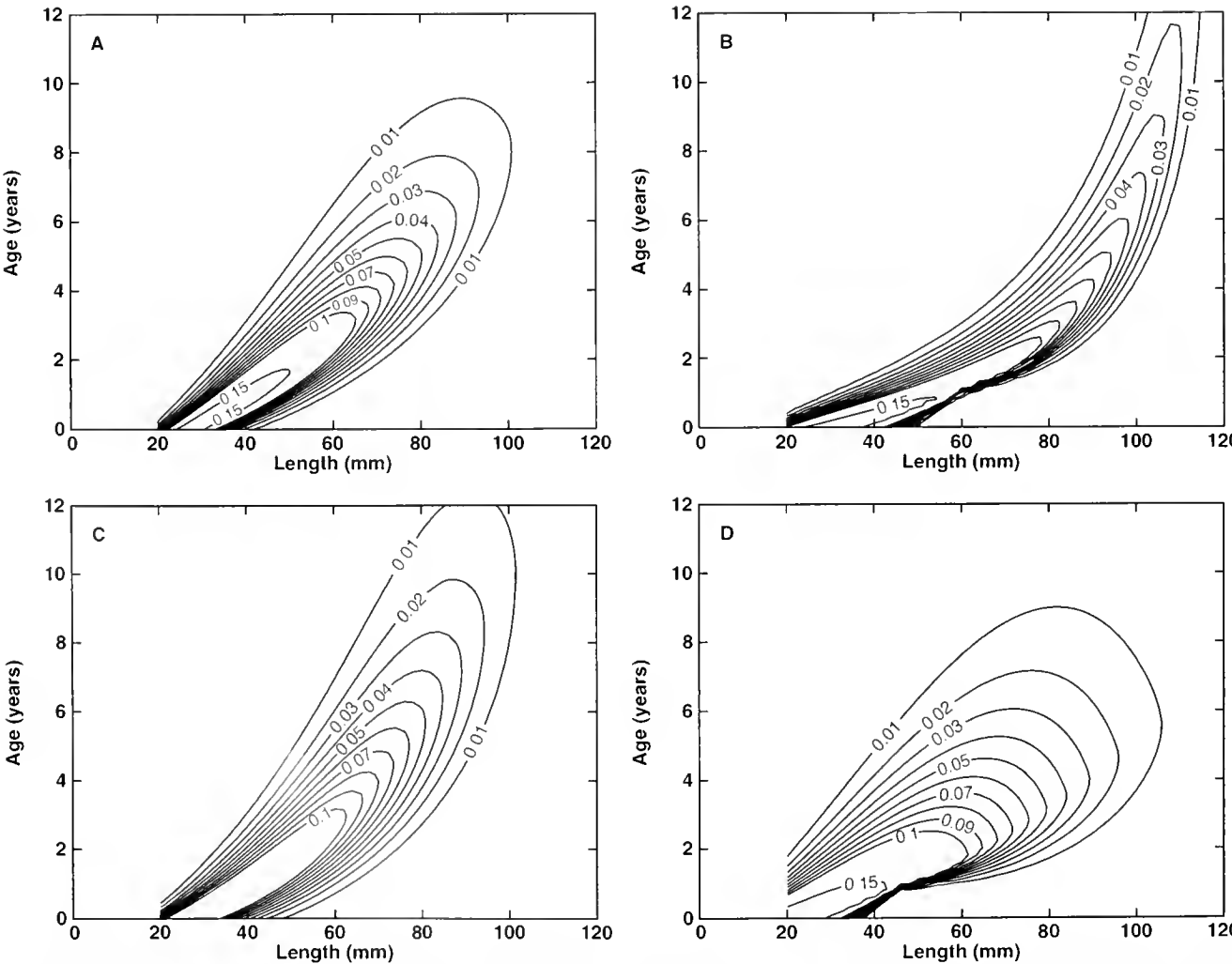


Figure 34. Age-length distributions obtained by fitting the twisted bivariate Gaussian formulation (Eq. 35) to the simulated age-length data sets obtained for a hard clam population in Great South Bay for: (A) the reference simulation, (B) increased growth rate, (C) decreased adult mortality rate and (D) even apportionment of genotypes, obtained by setting  $\sigma_{c1}$  and  $\sigma_{c2}$  in Eq. 17 equal to 0.25. Contours are number of individuals  $m^{-2}$ . The parameter values obtained from fitting the twisted bivariate Gaussian to the different simulated hard clam age-length data sets are given in Table 4.

The simulated age-length data sets produced with decreased growth rate, increased adult mortality, decreased adult mortality, and expanded genetic range resulted in optimal values for the central length ( $L_0$ ) that are smaller than that obtained for the reference simulation age-length data set (Table 4). The value of  $L_0$  determines where the  $x$ -axis of the rotated coordinate system intersects the length axis in the original coordinate system (Figs. 33 and 34). The value of this parameter indicates that the small animals have a smaller average size in these simulations than those in the age-length distributions obtained from the reference simulation. The age-length data sets from the increased growth-rate, reduced genetic range and decreased recruitment rate simulations gave an optimal  $L_0$  that is larger than the value obtained from the reference simulation. This indicates that the small animals in these simulations are on average larger than those in the reference simulation.

The age-length distribution created by decreased growth rate yields values for  $L_0$ ,  $a$ ,  $\sigma_L$  and  $b_2$  that are reduced relative to the values obtained from the reference simulation (Table 4). The parameter  $\theta$  is unusually high. The parameters  $\sigma_L$  and  $b_2$  produce greater asymmetry in the length and age-frequency distributions, which skews these distributions towards smaller lengths and older ages. These characteristics are consistent with a slow growing hard clam population. The negative value for  $a$  indicates a tendency for old animals to grow slower at a given length and the high value of  $\theta$  shows that age increases relatively rapidly for an increase in length. Collectively, no other simulated condition has values of  $L_0$ ,  $a$ ,  $\sigma_L$  and  $b_2$  simultaneously this low and  $\theta$  this high.

Increased growth rate creates a simulated hard clam population with an age-length distribution that is characterized by a combination of high  $L_0$ ,  $S_2$  and  $\sigma_L$  and low values for  $\theta$ ,  $a$  and negative  $b_2$ . These parameters result in a narrow, elongated age-length distribution that intersects the length axis at a small angle (Fig. 28A). The curvature of the age-length distribution produced with increased growth rate is more pronounced than that from other simulations and as a result, the parameter ( $n$ ) determining the curvature of  $y_m$  differs from the parabolic dependence obtained for the reference simulation and all other cases (Table 4). No other simulation has this unique set of parameters, the low values of  $b_2$  and  $a$  being particularly noteworthy.

A decrease in adult mortality yields high  $a$ ,  $\sigma_L$  and  $b_2$  values, which produce an age-length distribution that has more curvature in the long axis and increased spread in age for a given length (Fig. 29A). The high value of  $\sigma_L$  distinguishes this simulation from another simulation with high  $a$  and  $b_2$  values, the simulation of a decreased range of genotypes (Fig. 32A), and this can be observed in Figure 29A by the broader range of values on the rotated  $x$ -axis of this simulation. Increased adult mortality (Fig. 29B) results in an age-length distribution with low  $\sigma_L$  and  $S_1$  values (Table 4). This combination of parameters gives an age-length distribution that is less spread out about the rotated  $y$ -axis and the rotated  $x$ -axis than the distributions produced by other parameter combinations.

The parameter fits from the simulation with lower recruitment in the last five years of the simulation (Fig. 31A) yield a lower value of  $S_2$  and an exceptionally high value of  $\sigma_L$  relative to the reference simulation (Table 4). These parameters control the spread of the Gaussian about the rotated  $x$ -axis and reduce the asymmetry about the  $y$ -axis. The narrowing of the age-length distribution at small length is an expression of this parameterization.

Increasing the relative abundance of slow and fast growing clams to the composition of the cohort, by increasing the evenness of the distribution of genotypes in the cohort, modifies all of the parameters that control the spread of the age-length distribution

about the long axis (Table 4). This combination of parameters gives an age-length distribution that is straighter and spreads out asymmetrically at older age and longer lengths (Fig. 32B). Reducing the distribution of genotypes in a cohort again modifies the optimal parameter set (Table 4) and produces an age-length distribution that is narrow and symmetric with age and length (Fig. 32A). Note the unusually low values of  $b_1$  and  $\sigma_L$  is unusually low for both of these genotype modifications, indicating the increased uniformity in cohort composition, caused in one case by the increase in abundance of genotypes rare in the reference simulation and the other by the decrease in abundance of the same genotypes.

## DISCUSSION

### *Formulation of Model Processes*

The hard clam simulations provide guidance for future research topics because formulations used for some processes included in the model differ from those routinely used in population dynamics models. Hard clam mortality was assumed to be age, not length, dependent. The filtration rate parameterization provides an asymmetric increase with increasing temperature up to a specified temperature, after which it declines sharply. These parameterizations were needed to obtain simulated growth rates for individual hard clams that matched observed rates.

Additional assumptions were made that affect the simulated cohort and population structure. These include parameterizations that limit fecundity as individual clam length increases and impose a temperature- and time-dependent control on the reproductive cycle. The concept of quality days was used to terminate hard clam reproduction so that the duration of the simulated reproductive cycle and egg production matched observations. That hard clam reproduction does cease in the fall is known. However, the basic physiological processes that result in cessation of reproduction are not known. This is one area highlighted by the model development where more research is needed.

The form of the broodstock-recruitment relationship places a strong constraint on how the cohorts are concatenated to form a population. The form chosen for this study is one of many possible approaches (Kraeuter et al. 2005). Specification of the appropriate form requires continued and long-term monitoring of hard clam populations.

The simulations show that chlorophyll *a* alone is not adequate to support hard clam growth, which is consistent with results from models developed for other shellfish (Soniati et al. 1998, Hyun et al. 2001). Comparisons with observations from Great South Bay show that the hard clam growth form (e.g., timing and rate of growth) is not adequately simulated by a chlorophyll-only food source, implying that alternative food sources are being used. This required that the food supply input to the model be modified to allow for nonchlorophyll food sources (cf. Fig. 9D). The nature and magnitude of the alternative food sources needed by hard clams is deserving of investigation and may be an important component of hard clam restoration efforts.

The individual-based hard clam model was developed for Great South Bay where salinity and turbidity variations are small and do not typically extend into ranges that inhibit metabolic processes. Application of the hard clam model to environments characterized by lower salinity or higher total suspended solid concentrations than are typical for Great South Bay requires further verification studies. In particular, little experimental data exist on the effects of salinity and its interaction with temperature and total suspended solids on hard clam physiology and metabolism.

### Generalized Age-length Relationship

In individual shellfish, growth in age and length is partially decoupled. Growth rates within a cohort are such that many animals of differing ages may have the same length and many animals of differing length may have the same age. The age-length structure of a population is determined by a finite number of processes that control cohort length frequency, such as growth rate (Craig & Oertel 1966) and mortality rate, either of which may be age- or length-dependent. A small number of growth models routinely fit the length-age relationship of cohorts of a variety of species (e.g., Kappenman 1981, Kauffmann 1981, Tanabe 1988, Smith et al. 1997). The success of individual- and cohort-based growth models, such as the von Bertalanffy model, however, suggests that a few mathematical relationships might also describe the length-age relationships of populations. This study provides an example of the development of such a general relationship for hard clam populations.

Defining a general mathematical relationship that describes the age-length distributions allows development of age-length keys from fewer age-length measurements and application of age-length models. Moreover, population-to-population differences in the parameterization of the age-length model may be illustrative of important ecological and population dynamics processes determining the structure of the population just as variations in the parameters used in a von Bertalanffy growth model reflect differences in the structure of a cohort (e.g., Kornobis 1977, McCuaig & Green 1983, Nix et al. 1995).

### Implications of a Generalized Age-length Relationship

The earlier mentioned analyses suggest that a twisted bivariate Gaussian function can successfully describe age-length distributions that are produced by modifications in hard clam population dynamics, including changes in growth rate, mortality rate, recruitment rate and cohort genotypic composition. The age-length characteristics of populations have not been fully investigated since the initial development of a theoretical growth model (Pütter 1920, von Bertalanffy 1938) in spite of numerous experimental and theoretical studies at the cohort level. Thus, the extent to which the present formulation can be extended to other shellfish and non-shellfish taxa remains unclear. The robustness of the formulation described by the twisted bivariate Gaussian may have general application for producing age-length distributions.

The different population processes that were used to produce the simulated age-length data sets used to test the twisted bivariate Gaussian yielded a unique set of parameter values that defined the angle of the long axis, the degree of curvature, the length of the relation along the twisted  $x$  axis, and the tendency for the  $y$  axis to spread asymmetrically about  $x$  (Table 4). These characteristics of the age-length distribution are controlled by the growth rates, the form of and degree of mortality imposed on the population, the stability of recruitment, and the inherent genetic composition of the recruits. These parameters provide information concerning the processes controlling the structuring of the population in much the same way as values of growth parameters (e.g., the von Bertalanffy  $k$  and  $L_{\infty}$ ) provide information on the processes controlling cohort growth. The extent to which the specific values of the parameters might be predictive of certain combinations of population dynamics cannot yet be determined, but the simulations presented here are encouraging of such an outcome. Obtaining a set of ages and lengths and then fitting the twisted bivariate Gaussian to these data, could potentially provide more insight into population processes than may be obtained from mul-

tiyear studies of the suite of complex population dynamics-determining processes that produce observed age-frequency distributions.

Measurements of length are easy to obtain, but age is the more often desired quantity and conversion of the one into the other requires age-length keys, which are time consuming to construct. As a result, more is known about length frequencies in natural populations than about the complementary age frequencies. The cost and time of obtaining age-length keys directly limit their use in fisheries management and in ecological investigation. As a consequence, little is known about how age-length properties of populations might change within metapopulations, along environmental gradients, or in response to long-term climate changes. For long-lived animals such as hard clams, the latter is particularly important.

Generating an age-length key requires information from many individuals of differing ages for a given length and many individuals of differing lengths for a given age (Fig. 27). Because the age-length distributions twist along the length axis and spreads asymmetrically, the age-length array must be filled over all lengths and ages. As a result, a simple statistical function will not have the flexibility to describe the variation in age with length or in length with age over all ages and lengths. For example, a simple Gaussian function will accumulate increasing error as age and length increase. In the larger length classes and older age classes, the increased rarity of the animals themselves imposes an additional constraint on the function used to describe age-length distributions. As a consequence, filling age-length key arrays with sufficient replicate measurements to describe the underlying statistical distributions is difficult.

The availability of a single mathematical formulation to describe age-length distributions for hard clam populations provides a significant advance in the ability to generate age-length keys. Many fewer individual age-length measurements are necessary, provided they are adequate representations of the population, to describe the age-length distribution. A sparser data set might be used to determine parameter values for the general mathematical formulation that then can be used to fill in the full age-length distribution needed for a key. The density of the data over the age-length spectrum of the population needed to do this requires further investigation, but tests done with the simulated age-length data sets produced in this study suggest that age-length keys could be created with tens rather than hundreds of age-length measurements. Should this prove to be the case, then the existence of a general mathematical formulation provides opportunity to investigate how age and length independently determine population structure beyond what has heretofore been possible to achieve. Furthermore, the twisted bivariate Gaussian expands the theoretical formulations defining the age-length character of the cohort into that of the population by identifying parameters analogous to the rate of length increase ( $k$ ) and maximum length ( $L_{\infty}$ ) in the von Bertalanffy model (von Bertalanffy 1938, Fabens 1965, Kimura 1980). These parameters can now serve as the basis for theoretical models of population age-length compositions because they are determined by individual growth and mortality rates, but also by population-dependent processes, compensatory and otherwise.

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## RECRUITMENT PATTERNS AND PRECAUTIONARY EXPLOITATION RATES FOR GEODUCK (*PANOPEA ABRUPTA*) POPULATIONS IN BRITISH COLUMBIA

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**ABSTRACT** An age-structured projection model was used to study the impacts of alternative exploitation intensities on geoduck populations, based on a large accumulation of survey data, age composition data and published estimates of natural mortality. Data were analyzed and results presented by geographic region. Historic recruitment patterns were back calculated using an age-structured model. Trends in recruitment were independent of the value of  $M$  used, although rates were higher when  $M$  of 0.036 was applied compared with  $M$  of 0.016. Historic recruitment rates were found to be highly variable in the prefishery state: rates generally increased from the early 1930s to 1950, decreased until early 1960, increased to another peak in the mid 1960s, declined until the mid 1980s and have been increasing to present. Future recruitment was simulated from the estimated historic pattern. The fishing patterns examined were combinations of different mortality rates (0.016 or 0.036) and different periods of historical recruitment (beginning in 1940 or 1960). For each simulation year, in each set of 1,000 runs, the ratio of current biomass to virgin biomass was calculated and compared with the management objective of not exceeding 50% of virgin biomass within 50 y of harvest. An exploitation rate of 1.2% and 1.8% of estimated current biomass is recommended for the west coast of Vancouver Island and the rest of the coast respectively.

**KEY WORDS:** geoduck, *Panopea abrupta*, age, modeling, simulation, harvest rate, recruitment

### INTRODUCTION

Geoduck, *Panopea abrupta* (Conrad, 1849), are large hiatellid bivalves that thrive in unconsolidated substrates in the northern Pacific from California to southern Japan (Coan et al. 2000). The clams are extremely long-lived, with a maximum-recorded age of 168 y (Bureau et al. 2002). They are slow growing after an initial phase of fast growth during the first 10 y of life, and growth rate and maximum size varies along environmental gradients and between geographic regions (Hoffmann et al. 2000, Bureau et al. 2002). Geoducks start to reach sexual maturity at age 2–3 y (Campbell & Ming 2003). Reproduction is through broadcast spawning during early summer months, followed by pelagic larval stage lasting 40–50 days (Goodwin et al. 1979, Goodwin & Shaul 1984). Postlarvae settle and move on the seafloor surface for several weeks (King 1986) until metamorphosis and the start of suspension feeding. The animals begin to dig and reach their final refuge depth of up to 1 m in approximately 4–5 y, after which predation mortality is extremely low. An exception to low mortality is in regions where the sea otter (*Enhydra lutris*) populations are recovering.

Geoducks are aggregated into beds, by virtue of their substrate requirements, and are thus structured as metapopulations with segments being interconnected through the flow of planktonic larvae. No evidence has been found of a relationship between the reproductive capacity of a population within a given location and subsequent recruitment to that location. Recruitment mechanisms operate differently during the periods of pre settlement and post settlement of larvae, and Orensanz et al. (2004) warn that key processes in the population dynamics may be completely blurred if analyzed at the wrong spatial scale.

Geoduck populations support lucrative dive fisheries in Washington State and British Columbia (BC), with an average annual landed value of 38 million Canadian dollars over the last five years in BC. The BC fishery is managed through a combination of limited entry and individual quotas (Heizer 2000). Annual quotas

are set at 1% of estimated virgin biomass, the harvest rate being a result of initial yield models that were based on intuition and the limited biological information available at an early stage of fishery development. To date, virgin biomass has been estimated by adding fishery removals to the estimate of current biomass from transect surveys, while natural mortality and recruitment are assumed to be equal. In contrast, the harvest rate in Washington is set to be 2.7% of current biomass based on the  $F_{40\%}$  criterion (Bradbury & Tagart 2000).

The availability of a substantial accumulation of biological information warrants a new assessment of the geoduck stocks and re-evaluation of harvest rates in BC. Fishery-independent transect surveys have been conducted over all regions of the BC coast and, to date, include about 40% of the total estimated area of geoduck beds. Geoduck abundance, distribution and age composition have been collected from each surveyed location. Some important biological parameters of geoduck populations, such as growth and natural mortality rates, have also been estimated (Bureau et al. 2002, Zhang & Campbell 2004). In this paper, we study historic recruitment patterns through back-calculation, and investigate the impacts of alternative exploitation intensities on the geoduck populations through age-structured projection modeling.

### MATERIALS AND METHODS

We conducted this study using biological information obtained from fishery-independent surveys and sampling, and fishery data obtained from logbooks. We back-calculated historic recruitment patterns using an age-structured model, and simulated future recruitment from the estimated historic recruitment pattern. The accuracy of the simulations was examined by comparing projected abundances with observed ones in four instances where repeat survey data were available. Through forward simulation, we evaluated the impacts of alternative fishing intensities on the stocks. Data were analyzed by survey bed and the results grouped by geographic region. In this paper, we divide the BC coast into five geographic regions: North Coast (NC), Central Coast (CC), Queen Charlotte Islands (QCI), West Coast of Vancouver Island (WCVI)

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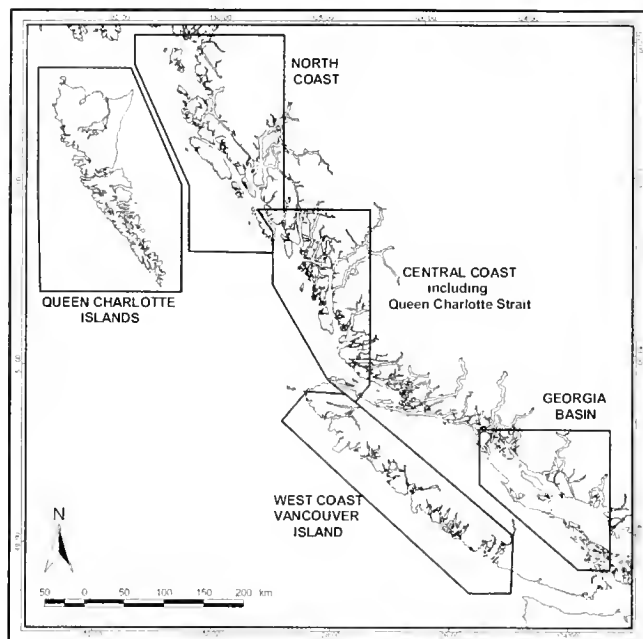


Figure 1. Five geographic regions in the British Columbia coast.

and Georgia Basin (GB) (Fig. 1). In this study, CC also includes two surveyed locations in Queen Charlotte Strait.

#### Fishery-independent Surveys

Each year, a number of geoduck beds are selected for surveying. Beds are chosen for various reasons, including significant contributions to total landings in the fishery, disagreement in the recommended quota for a bed or conservation concerns. If the selected location is comprised of many smaller beds, as opposed to beds that occur on large banks, the aggregate is partitioned into strata. For each strata, transect locations are selected randomly, following protocols described in Campbell et al. (1998) and Babuin et al. (2006). A number of quadrats 2-m wide and 10-m long are set along each transect from 60 feet chart datum to the intertidal, and SCUBA divers count the number of geoduck within each. Where appropriate, data are poststratified for analysis, where individual transects are excluded if the substrate was not suitable. Mean geoduck density and associated standard error were estimated for each bed using the two-stage random design estimator with transects as the primary sampling units and quadrats as the secondary sampling units (Thompson 1992). Biological samples are collected from the surveyed populations, from which length, weight and age data are obtained (Bureau et al. 2002, 2003). Age is determined using the acetate peel method (Shaul & Goodwin 1982) and, in most cases, estimated geoduck ages are provided with a range of uncertainty. Subsamples of approximately 150 animals each are collected from randomly selected transects throughout the survey location and combined into one sample per location.

Density and biological information from 42 fishery-independent surveys was used in the modeling. The 42 surveys were conducted in 38 locations; 4 locations were surveyed in two different years (Table 1). The number of individual geoduck beds surveyed per location varies from 1 to 18 (Table 1). From each surveyed location, between 186 and 580 geoduck were sampled for age determination.

#### Estimation of Historical Recruitments

Recruitment age is defined to be six years, the age at which we assume geoducks to become fully vulnerable to harvesting (Campbell et al. 2004). Historical recruitment rates (number of recruits/ $m^2$ ) were back calculated for each survey location, using estimated geoduck density, the age structure of the bio-samples collected and fishery removals as inputs to the model. Estimated historic recruitment strength relies on the natural mortality rate,  $M$ , used in the back-calculation.  $M$  for geoduck populations appears to vary approximately between 0.01 and 0.04 (Breen & Shields 1983, Harbo et al. 1983, Sloan & Robinson 1984, Noakes & Campbell 1992, Bradbury & Tagart 2000). In a tagging experiment in Washington,  $M$  was estimated to be 0.016 with a standard error of 0.0046 (Bradbury et al. 2000). Using experimental data from WCVI, Zhang and Campbell (2004) estimated  $M$  to be 0.036 with a standard error of 0.003. These two estimates of  $M$  approximately cover the range of  $M$  found in the literature, and were used in this study to estimate historic recruitment patterns.

The youngest age considered in this paper is six years. The maximum age was set to be 80 y for the sampling year, and geoduck older than the maximum age were pooled to form a maximum-plus age group. The maximum age for previous years was one year younger than for the subsequent year. For instance, in the year prior to the sampling year, the maximum age was 79 y and geoduck older than age 79 were pooled into the 79 + age group. In the sampling year, the abundance of geoducks for each surveyed bed is calculated as the product of mean density and the bed area, and the total abundance for each surveyed location is simply the sum of abundances for all the surveyed beds in the location. For each location, abundance at each age during the sampling year was estimated as the product of the total geoduck abundance and the proportion of geoduck at that age in the bio-sample. Abundance of geoduck at age  $a$  in year  $y$  (earlier than the sampling year),  $N_{y,a}$ , was estimated as:

$$N_{y,a} = N_{y+1,a+1} \times \exp(M) + C_{y+1} \times P_{y+1,a+1} \quad (1)$$

where  $C_{y+1}$  is the catch in number of geoduck in year  $y + 1$ , and  $P_{y+1,a+1}$  is the estimated proportion of geoduck at age  $a + 1$  in year  $y + 1$ . Thus, the proportion-at-age in the bio-samples was carried backward in the retrospective analysis as the proportion at each age-class. Where commercial landings were only reported by weight,  $C_{y+1}$  was estimated by dividing the landed weight by the mean geoduck weight for the location. Mean weight for a bed is estimated from the landed weight and number of geoduck, where number is reported in logbooks, over the period 1997 to present. Mean weight estimates from nearby beds may be applied if no estimate for a given bed exists.

Annual recruitment rate for an individual surveyed location was expressed as recruitment density by dividing the estimated abundance of age-6 in each year by the summed area over all the surveyed beds. Annual recruitment rate for a geographic region was calculated as:

$$R_y = \frac{\sum_{\text{location}} N_{y,6}}{\sum_{\text{location}} A} \quad (2)$$

where  $N_{y,6}$  represents the estimated abundance of age-6 geoducks in year  $y$  in a location, and  $A$  represents the summed area over all the surveyed beds in a location.

TABLE 1.  
Information on fishery-independent surveys.

Geographic Region <sup>1</sup>	Survey Location	Fishery Starting Year	Survey and Sampling Year	Number of Beds	Biological Sample Size
CC	Anderson/Laredo	1987	1997	10	293
CC	Goose/Wurtele/Seaforth	1981	1995	9	460
CC	Hakai Passage	1982	1998	14	292
CC	Kitasu Bay <sup>2</sup>	1989	1994	5	434
CC	Price Island	1987	1993	3	455
CC	S Bardswell/Prince Group	1985	1996	11	427
CC	West Higgins Pass <sup>2</sup>	1985	1994	2	474
CC	Duncan Island	1984	1995	13	468
CC	Goletas Channel, 1994 <sup>2</sup>	1984	1994	5	447
CC	Goletas Channel, 2003	1984	2003	8	459
NC	Dundas Island	1986	1998	6	306
NC	Moore Islands	1996	1998	4	290
NC	Otter Pass	1981	1996	6	427
NC	Principe Channel	1996	1997	7	298
NC	West Arstazabal Island	1983	1996	15	395
QCI	Burnaby Island	1984	1994	7	431
QCI	Cumshewa Inlet	1980	1997	7	480
QCI	Gowgaia Bay <sup>2</sup>	1985	1999	8	270
QCI	Hippa Island <sup>2</sup>	1986	1999	6	432
QCI	Hotspring Island	1986	1995	10	385
QCI	Houston Stewart Ch.	1985	1996	14	453
QCI	Parry Passage	1987	2002	1	440
QCI	Selwyn/Dana/Logan Inlets	1988	1998	18	321
QCI	Tasu Sound <sup>2</sup>	2000	2001	12	446
GB	Boatswain Bank	1980	2001	1	536
GB	Comox Bar, 1993	1978	1993	1	440
GB	Comox Bar, 1998	1978	1998	1	289
GB	Marina Island	1978	2002	2	304
GB	Oyster River	1978	1996	1	466
GB	Round Island	1979	2000	1	322
GB	Thormanby Island	1978	1999	2	283
WCVI	Barkley Sound	1979	2000	9	301
WCVI	Elbow Bank	1978	1994	1	405
WCVI	Millar Channel	1979	1997	1	277
WCVI	Mission Group, 1998	1980	1998	5	304
WCVI	Mission Group, 2003 <sup>3</sup>	1980	2003	7	456
WCVI	NE Barkley Sound	1982	2002	8	501
WCVI	Nootka Sound <sup>3</sup>	1985	2000	7	311
WCVI	Rolling Roadstead <sup>2,3</sup>	1980	2001	1	418
WCVI	Winter Harbour, 1996	1983	1996	10	580
WCVI	Winter Harbour, 2002 <sup>3</sup>	1983	2002	6	495
WCVI	Yellow Bank	1980	1997	1	186

<sup>1</sup> Central Coast (CC), North Coast (NC), Queen Charlotte Island (QCI), Georgia Basin (GB), West Coast of Vancouver Island (WCVI).

<sup>2</sup> Biological sampling was completed one year after the transect survey, for logistical reasons.

<sup>3</sup> Surveyed locations where geoduck populations have been impacted by sea otters.

To evaluate uncertainties in the estimation, 999 simulations were conducted. In each simulation, geoduck densities, natural mortality rates, and age compositions were regenerated, and the simulated data sets were used to estimate the recruitment patterns, as described above. Densities in the sampling year were randomly regenerated from a normal distribution with the mean and standard deviation estimated from survey results. The value of  $M$  was randomly regenerated from a normal distribution with the designated mean and standard error ( $0.016 \pm 0.0046$  or  $0.036 \pm 0.003$ ). The starting age composition of the bio-samples was bootstrapped by resampling with replacement to produce a simulated sample of the same size. A further step incorporated the uncertainty in ageing;

the actual age for each sample was randomly generated from a normal distribution, with the estimated age as the mean and one half of the error-range as the standard deviation. To test the impact of ageing uncertainty on simulated biomass estimates, we compared the expected biomass at the end of the simulation years with and without this uncertainty.

#### Projection Simulations

We assume that recruitment in the future will reflect what has occurred in the past. We fitted the 1,000 back-calculated recruitment data to the gamma probability distribution for each surveyed location using the least square error method, and used the fitted

distribution to describe the likelihood of recruitment rates for the location. In each simulation year, the number of recruits was randomly generated from the gamma distribution. For each surveyed location, biomass was projected onwards for 50 y from the sampling year under a given exploitation rate ( $E$ ), which varies from 0% to 4% with an interval of 0.5%. We examined the impact of alternative harvesting intensities on the surveyed stock biomass to find plausible and precautionary  $E$ 's for the fishery. Population abundance at age  $a + 1$  in year  $y + 1$  was estimated to be:

$$\begin{cases} N_{y+1,a+1} = N_{y,a} \times (1 - E) \times \exp(-M) & (a < 80) \\ N_{y+1,a+} = (N_{y,a} + N_{y,a+}) \times (1 - E) \exp(-M) & (a = 80) \end{cases} \quad (3)$$

We used the weight-age conversion equations, which were established for each surveyed location by Bureau et al. (2002, 2003), to convert abundance to biomass. For each simulation year, a performance index was calculated as the ratio of current biomass ( $B_c$ ) to the virgin biomass ( $B_v$ ), the latter being the back-calculated biomass in the year just before the beginning of the commercial fishery in the location. The performance index for each geographic region was calculated by dividing the sum of  $B_c$  by the sum of  $B_v$  over all the surveyed locations in the geographic region. Among the nine surveyed locations in WCVI, four have been affected by sea otter predation (Table 1). We therefore calculated the ratio of  $B_c$  to  $B_v$  by using only the five unaffected locations.

Four possible simulation schemes were considered: recruitment simulation year starts from either 1940 or 1960, and the mean value of  $M$  used in the simulation is either 0.016 or 0.036. The 4 simulation schemes are thus denoted as Y40M0.016, Y60M0.016, Y40M0.036 and Y60M0.036. In general, future recruitment is more optimistic when the recruitment simulation year starts in 1940 than in 1960 because estimated recruitment is high during the period 1940–1960.

To evaluate uncertainties in the estimation, 999 simulations were conducted. In each simulation, age composition of the bio-samples, geoduck density in the sampling year, and value of  $M$  were randomly regenerated as described in the previous section.

#### Comparison of Projected and Survey-Derived Geoduck Densities

Surveys were conducted in two different years in each of 4 locations: Goletas Channel, Winter Harbour, Mission Group and Comox Bar (Table 1). This provides an opportunity to examine the location of the observed mean density within the range of the

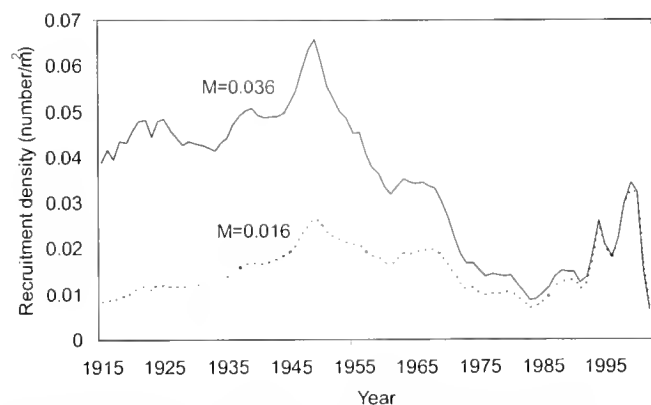


Figure 2. Estimated mean historic recruitment rates for the entire BC coast.

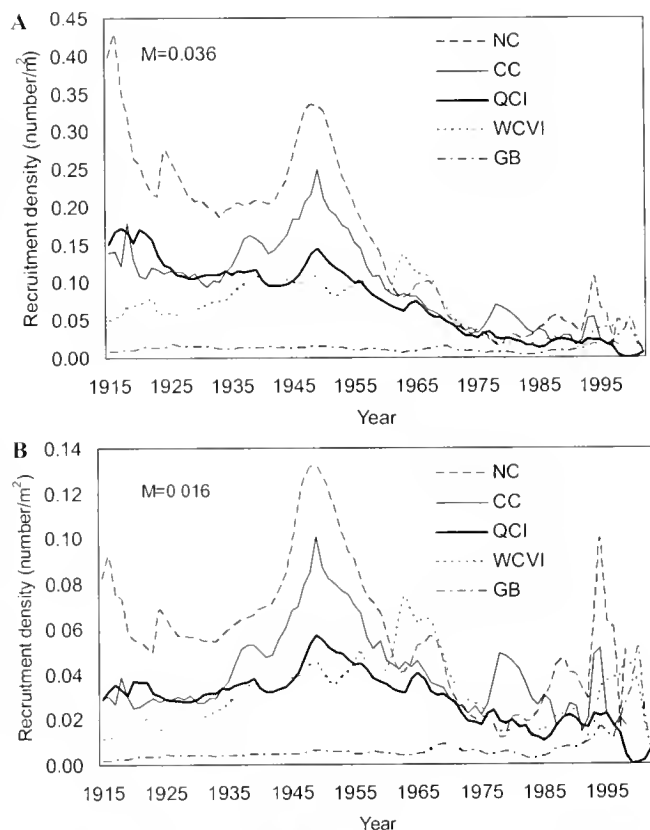


Figure 3. Estimated mean historic recruitment rates for the five geographic regions: North Coast (NC), Central Coast (CC), Queen Charlotte Islands (QCI), West Coast of Vancouver Island (WCVI), and Georgia Basin (GB).

model-projected densities, which is helpful in selecting a percentile level for determining harvest rates. Except in Comox Bar, different sets of the geoduck beds were surveyed on the two occasions; we used only the data obtained from beds which were surveyed on both occasions. We used results from the first survey to predict geoduck densities in the year of second survey. In each of the simulation years, recruitment was randomly generated from the gamma distribution, as described earlier. Population abundance

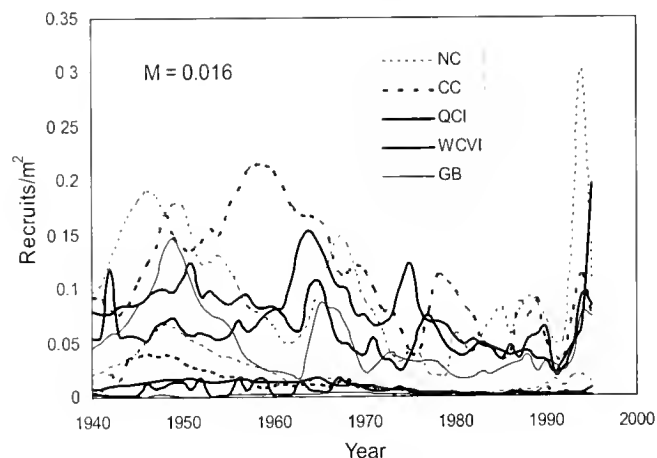


Figure 4. Comparison of the estimated range of mean historical recruitment rates for individual locations within each of the five geographic regions ( $M = 0.016$ ).

TABLE 2.

Exploitation rates to maintain stock biomass above one-half of the virgin biomass level at the end of 50 years for the five geographic regions: North Coast (NC), Central Coast (CC), West Coast of Vancouver Island (WCVI), Queen Charlotte Island (QCI) and Georgia Basin (GB).

Natural Mortality Rate	Beginning Year of Recruitment Simulation	Percentile	Geographic Region					
			NC	CC	WCVI	WCVI*	QCI	GB
0.036	1940	10th	1.7%	3.0%	2.2%	2.0%	2.0%	2.4%
		50th	2.5%	3.6%	3.0%	3.0%	2.6%	3.3%
	1960	10th	0.0%	0.0%	1.1%	1.2%	0.0%	2.0%
		50th	0.0%	0.3%	1.7%	1.9%	0.2%	2.7%
0.016	1940	10th	2.0%	3.0%	2.9%	2.9%	2.4%	3.7%
		50th	2.4%	3.3%	3.4%	3.6%	2.7%	4.0%
	1960	10th	1.1%	1.8%	2.8%	2.8%	1.6%	4.0%
		50th	1.3%	2.0%	3.3%	3.5%	1.8%	4.0%

\* Only include the surveyed locations with absence of sea otter.

at age  $a + 1$  in year  $y + 1$ ,  $N_{y+1,a+1}$ , was calculated by rearranging Eq. 1. To incorporate uncertainties in estimated parameter values, 999 simulations were conducted.

## RESULTS

### Historic Recruitment Pattern

Trends in recruitment over time are comparable, irrespective of the value of  $M$  used in the back-calculation (Fig. 2), whereas the magnitude of estimated historic recruitment rates are affected by the value of  $M$ . Recruitment rates in the past were estimated to be higher when  $M$  of 0.036 was applied compared with a value of 0.016 (Fig. 2). Recruitment seems to have fluctuated appreciably in the virgin state in years prior to 1975. Overall, recruitment in the BC coast increased from at least 1930, and reached the highest around 1950. It then decreased until the early 1960s, before it started to increase again. Recruitment reached another peak in the mid-1960s, and then declined until mid-1980s. Recruitment has been increasing since the mid-1980s. In general, recruitment rates in 1940 to 1960 constituted the highest peak, and recruitment rates in 1960 to 1975 constituted a second, lower, peak. With application of  $M$  of 0.016, recruitment rates in recent years seem to be as high as in the highest historic period of 1940–1960. With application of  $M$  of 0.036, recruitment rates in recent years seem to be approaching the lower level in 1960–1975. The apparent decline in the most recent years is most likely because of under-sampling of young geoduck in the bio-samples.

Among the five geographic regions, average recruitment rates are the highest in the North Coast (NC) and Central Coast (CC), intermediate in Queen Charlotte Islands (QCI) and West Coast Vancouver Island (WCVI), and the lowest in Georgia Basin (GB) (Fig. 3). Recruitment patterns in NC, CC and QCI generally conform to the overall pattern for the entire coast whereas recruitment patterns in WCVI and GB lack the highest peak around 1950 (Fig. 3). Recruitment rates in GB in recent years appear to be the highest for the period studied.

Despite apparent differences in the overall mean recruitment rates between most geographic regions, ranges of mean recruitment rates (extent of minimum and maximum mean recruitment rates) over individual locations within a region are wide, and there are substantial amounts of overlap in these mean recruitment rates (Fig. 4).

### Projection Simulations

For a given exploitation rate, stock biomass generally declines more quickly or increases more slowly, when the recruitment simulation starting year is 1960 rather than 1940, and/or when  $M$

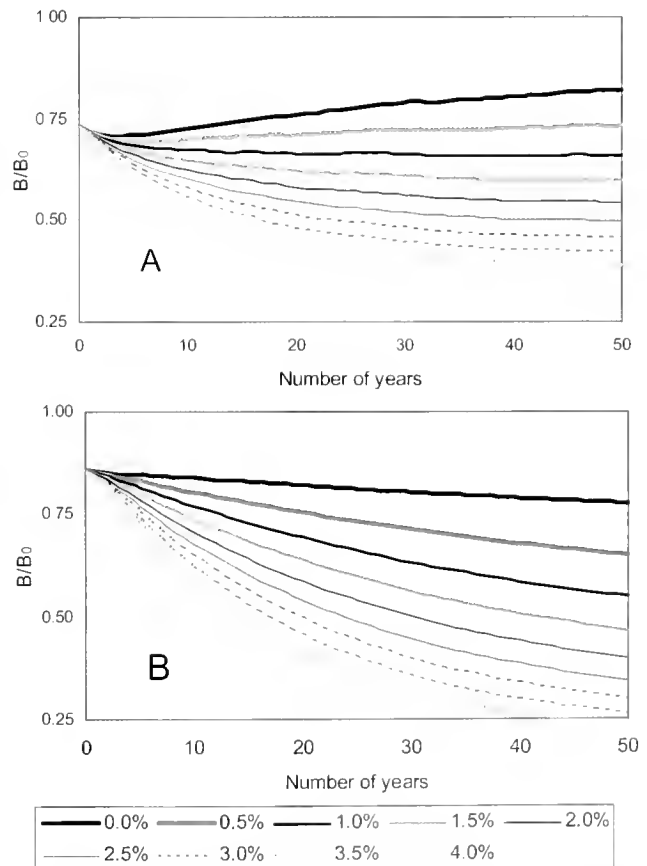


Figure 5. Simulated changes in the ratio of current biomass ( $B$ ) to the virgin biomass ( $B_0$ ) in the North Coast at the 50th percentile with different exploitation rates. (A: Recruitment simulation starting year is 1940 and  $M$  is 0.036. Note that biomass would decline to 50% of the virgin biomass with an exploitation rate of 2.5%. B: Recruitment simulation starting year is 1960 and  $M$  is 0.016. Note that biomass would decline even without harvesting).

used in the modeling is 0.036 rather than 0.016. All projection results are presented in Table 2.

With Y40M0.036, the geoduck stocks in NC could be harvested at 2.5% to have a 50% calculated chance of achieving the management goal of maintaining the biomass above one-half of the virgin biomass at the end of 50 y (Fig. 5A).  $E$  would be reduced to 1.7% if we want to raise the chance of achieving the goal to 90%. For the other four geographic regions,  $E$  could be set between 2.6% and 3.6% to have a 50% calculated chance of achieving the management goal, and would be reduced to 2.0% to 3.0%, if we want to increase the chance to 90% (Table 2). With Y60M0.036, the population would decrease in NC, CC, and QCI even without exploitation, because recruitment would not be sufficient to compensate for the loss caused by natural mortality. The geoduck stocks on WCVI could be harvested at 1.7% to have a 50% calculated chance of achieving the management goal of maintaining the biomass above one-half of the virgin biomass at the end of 50 y.  $E$  would be reduced to 1.1% if we want to raise the chance of achieving the goal to 90%. When we consider only the areas without sea otter predation, the corresponding  $E$ 's would be 1.9% and 1.2%. The geoduck stocks in GB could be harvested at 2.7% to have a 50% calculated chance of achieving the management goal, and 2.0% if we want to raise the chance to 90%. With Y40M0.016,  $E$  could be set to 2.4% to 3.6% for NC, CC, QCI and WCVI to have a 50% calculated chance of achieving the management goal and would be reduced to 2.0% to 3.0% if we want the calculated chance to increase to 90%. Stocks in GB again seem to be able to sustain higher harvest rates at 4.0%

and 3.7% for 50% and 10% percentiles, respectively. With Y60M0.016, recruitment would not be sufficient to compensate for the loss caused by natural mortality in NC (Fig. 5B), although it would take 50 y to reduce the biomass to one-half of the virgin biomass at a harvest rate of 1.3% (Table 2).  $E$  could be set at 1.8% to 3.5% to have a 50% calculated chance to achieve the management goal for CC, QCI, and WCVI and would be reduced to 1.6% to 2.8%, if we want to increase the chance to 90%. Geoduck stocks in GB seem to be able to sustain an exploitation rate around 4.0%.

Incorporation of uncertainty in geoduck ageing into the simulations produced slightly lower biomass estimates at the end of 50 y. The ratio of projected biomass to virgin biomass is, on average, 2.3% and 2.4% lower at the 50th and 10th percentile levels, respectively.

#### Comparisons of Recruitment Rates and Projected Densities

Recruitment densities estimated from the two independent surveys conducted in four locations agree with each other reasonably well (Fig. 6). Ninety-five percent, 82%, 92% and 87% of mean recruitment rates estimated from the second survey are located within the 95% confidence bounds based on the first survey for Goletas Channel, Winter Harbour, Mission Group and Comox Bar, respectively.

In Goletas Channel and Winter Harbour, the mean densities derived from the second surveys are either well within the interval of 10th to 90th percentiles or close to the 10th percentiles of

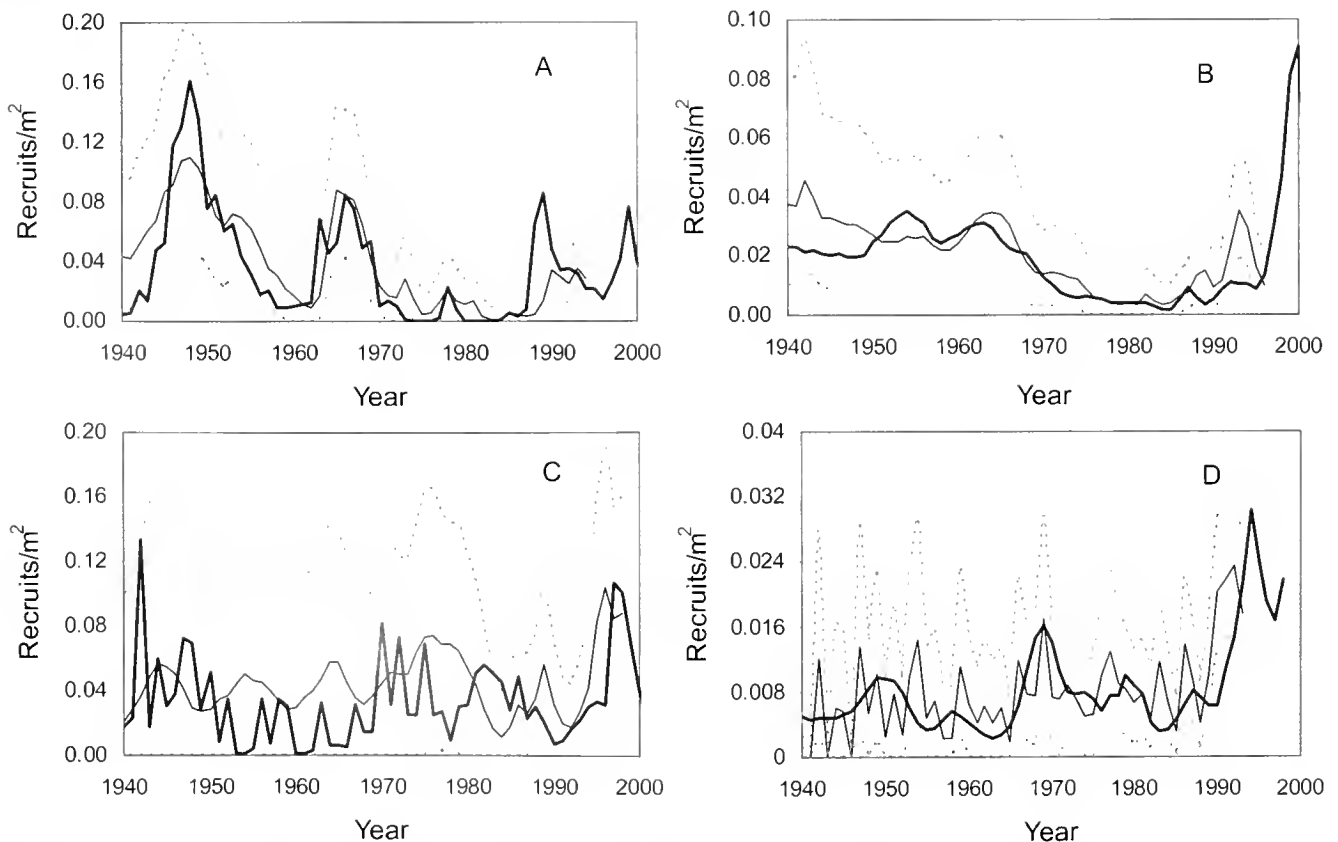


Figure 6. Comparison of recruitment density estimated from two surveys in different years ( $M = 0.016$ ). The solid line represents the mean estimate based on the first survey, and the broken lines denote the 95% confidence limits. The bold line represents the mean estimate based on the second survey. (A, Goletas Channel; B, Winter Harbour; C, Mission Group; D, Comox Bar).



projected densities based on the first survey (Fig. 7A, B). In Mission Group, the observed mean density from the second survey is considerably lower than the 10th percentiles (Fig. 7C), probably because a substantial amount of geoduck were consumed by sea otters. Sea otters are also known to reside in Winter Harbour, but their impact on geoduck appears to have been considerably less severe (DFO, unpublished data). In Comox Bar, the mean density derived from the second survey is close to or above the 90th percentiles (Fig. 7D), which is consistent with the particularly strong recruitment observed in GB in the 1990s (Fig. 3). Because recruitment simulation relies on estimated recruitment in the past, the model is incapable of accurately projecting the abundance or biomass trends when there is a persistence of especially high or low recruitment.

## DISCUSSION

This study provides a revealing glimpse at historic recruitment trends for geoduck populations in BC, and presents a method of evaluating impacts of alternative harvesting intensities on the geoduck stocks. Features related to the study and implications of our findings to the assessment and management of the geoduck fishery are discussed.

Geoduck stocks form metapopulations. There is virtually no stock-recruitment relationship in the traditional sense, because recruitment to one location is unlikely associated with the reproductive capacity in the same location (Orensanz et al. 2004). Compensatory or depensatory density-dependent processes were not explicitly incorporated into the analyses because there is no clear evidence of these mechanisms having an impact on recruitment,

survival and growth (Orensanz et al. 2004). Unless and until we could model the larval movement, we are not able to link recruitment in one spot to stock biomass in another. We are also currently unable to predict future recruitment rates based on environmental factors, although it is recognized that recruitment is likely related to the geographic and oceanic features in the immediate area. For instance, recruitment is higher in locations where water currents are of medium velocity (Goodwin 1990, Bureau et al. 2002). To conduct the recruitment simulations, we follow the advice of Maunder and Driso (2003) and assume that future recruitment will occur with a similar distribution as historical recruitment. Positive relationships between model-projected and observed abundances suggest that this approach is reasonable for geoduck populations in the absence of extraordinary recruitment or predation events.

Our estimated recruitment patterns for the five geographic regions generally agree with those reported by (Orensanz et al. 2004). Recruitment appears to have been strong in recent years throughout the BC coast, likely because of some favorable environmental conditions prevailing in these years (Valero et al. 2004). Overall historic recruitment trends appear to be similar for NC, CC, QCI, and, to a large extent, WCVI and GB as well, suggesting that geoduck recruitment may be regulated by common oceanic factors. However, within each geographic region, recruitment variation is large, which may preclude using regional index sites to represent recruitment trends in any given area.

Geoduck are long-lived animals, and natural mortality for geoduck populations must, therefore, be low.  $M$  has been estimated in the approximate range of 0.01–0.04 (Bradbury & Tagart 2000, Breen & Shields 1983, Noakes & Campbell 1992, Orensanz et al.

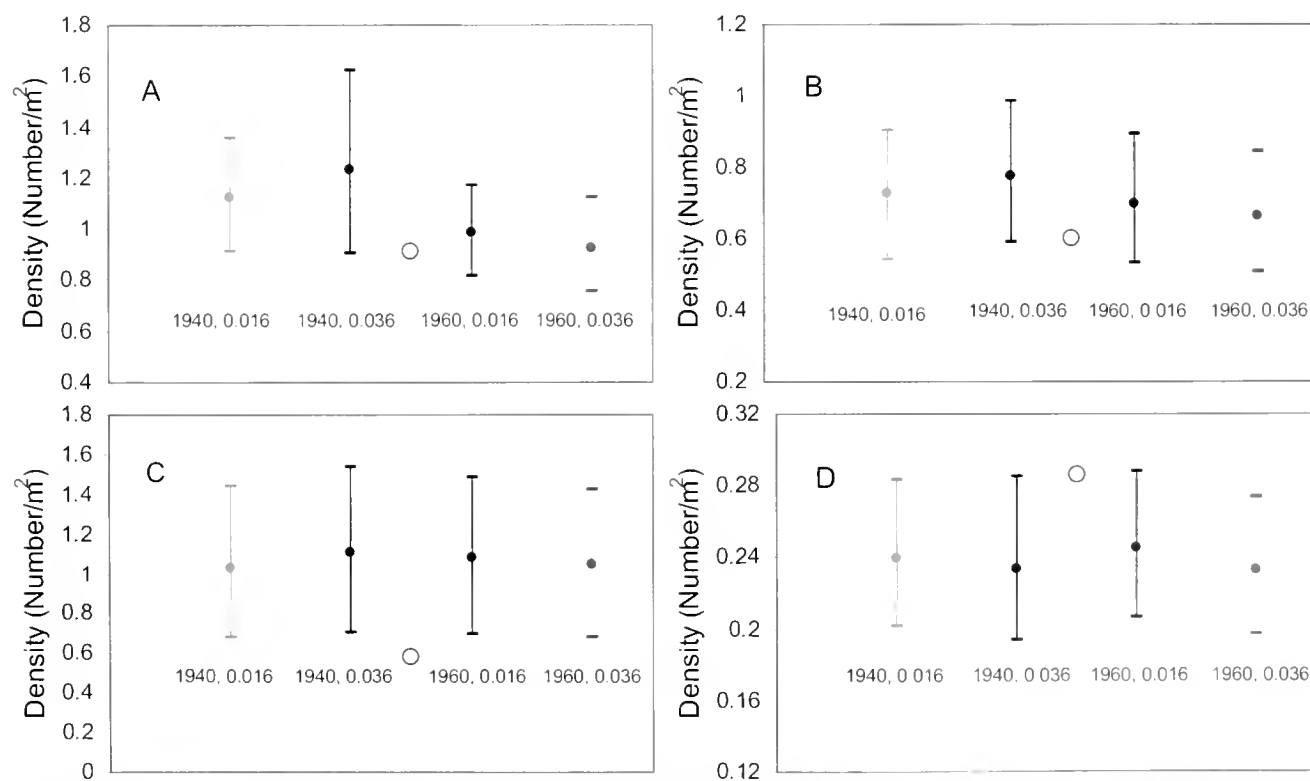


Figure 7. Comparison of projected and survey-derived geoduck densities. The solid dots represent the predicted median densities and the two bars represent 10th and 90th percentiles. The hollow circles denote the mean densities derived from the 2nd survey. Each set of two numbers indicates the starting recruitment simulation year and natural mortality rate used in the modeling. (A, Goletas Channel; B, Winter Harbour; C, Mission Group; D, Comox Bar).

2004, Zhang & Campbell 2004). We chose to use a small value of  $M$  (0.016) and a large value of  $M$  (0.036) in the simulation model to evaluate impacts of fishing intensities on the stocks. As actual  $M$  is likely to vary between the two values, the real impact of fishing intensities on stocks is likely to be between the two estimated extremes. Thus, choosing the more conservative exploitation rate from within the range provides for precautionary management. There is reason, however, to regard 0.036 as a more realistic average for geoducks than 0.016, which may to some degree underestimate the true lower range for  $M$ . The lower estimate came from a single tagging experiment on large, visible-siphon adult animals and might represent  $M$  for a lower-mortality portion of the population. In contrast, the higher estimate of  $M$  was developed using back-calculated age data over different sites and years, and therefore represents more than a single year of natural mortality and more than one population.

The productivity of a geoduck population is regulated by the balance of recruitment and growth on one side and natural mortality on the other. The rate of change in the ratio of current biomass to the virgin biomass depends on the productivity. The simulation model generates a wide range of exploitation rates over the simulation schemes, each rate having an associated calculated risk of failing the management goal. The comparison of model-projected and survey-derived densities in locations that were surveyed on two separate occasions indicates that the 10th percentile is a precautionary choice and more appropriate than the mean or median exploitation rates.

Simulation schemes that would cause the population abundance to decrease even without harvesting were considered to be unrealistic, particularly in areas where predation by sea otters has not been reported. Whereas some geoduck populations may decrease naturally in the short to medium term, particularly in areas that receive infrequent recruitment, modeling scenarios that lead to long-term stock collapse in the absence of harvest are not considered practical for commercial fishery management and are not considered further. The most conservative exploitation rates were generated from Y40M0.036 for the North Coast, from Y60M0.016 for Central Coast and Queen Charlotte Islands, and Y60M0.036 for West Coast Vancouver Island and Georgia Basin. Based on these precautionary simulation schemes, exploitation rates would be 1.7%, 1.8%, 1.6%, 1.2% and 2.0% for NC, CC, QCI, WCVI and GB, respectively. The proposed harvest rates for geoduck populations in BC are lower than the rate of 2.7% applied in the State of Washington, which was calculated based on the  $F_{40\%}$  criterion (Bradbury & Tagart 2000).

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## DIFFERENCES OF TURKISH CLAM (*RUDITAPES DECUSSATES*) AND MANILA CLAM (*RUDITAPES PHILIPPINARUM*) ACCORDING TO THEIR PROXIMATE COMPOSITION AND HEAVY METAL CONTENTS

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**ABSTRACT** The aim of this study is to determine the proximate composition, biometrical measurements and heavy metal content differences of marine bivalve *Ruditapes philippinarum* (Venice, Italy) and *Ruditapes philippinarum* (Izmir, Turkey). No statistical ( $P < 0.05$ ) difference was observed in the values of crude lipid, moisture, ash and carbohydrate but statistical differences occurred in their crude protein values. They were  $0.98 \pm 0.16\%$ ;  $83.98 \pm 0.16\%$ ;  $3.3 \pm 0.424\%$ ;  $1.14 \pm 0.02\%$ ;  $9.56 \pm 0.06\%$  for *Ruditapes decussatus*, respectively. They were  $0.78 \pm 0.16\%$ ;  $85.91 \pm 1.22\%$ ;  $3.1 \pm 0.232\%$ ;  $1.10 \pm 0.02\%$ ;  $8.71 \pm 0.18\%$ , for *Ruditapes philippinarum*, respectively. Each of the clam was determined as fresh and in perfect quality according to their TVB-N values but in the comparison of color values statistical difference was observed ( $P > 0.05$ ). Trace metal levels were below the legal limits set by national standards of Turkish governments.

**KEY WORDS:** clam, color value, proximate composition, *Ruditapes*, measurements, heavy metals

### INTRODUCTION

Water pollution has increased in heavy industrialized areas. Most industrial water wastes end up in the sea. Monitoring the elemental composition in marine organisms, such as clams, provides the essential elements in living organisms and through the food chain to humans. Bivalves can accumulate microorganisms, including pathogenic bacteria and viruses, when grown in polluted waters and can present a significant health risk when consumed raw or lightly cooked (West & Coleman 1986, Rippey 1994, Sobsey & Jaykus 1999, Croci et al. 2002, Formiga-Cruz et al. 2003). Pathogenic bacteria, such as *Salmonella typhi*, *Vibrio parahaemolyticus* and *V. cholera* remain viable for weeks in the marine environment, whereas viruses can survive for months in the same environment as well as in fish tissue. Some viruses, for example, the infectious hepatitis A virus, which has caused many shellfish associated outbreaks, can survive in seawater for more than a year and is frequently transmitted by shellfish consumption (Le Guyader et al. 2000, Muniain-Mujika et al. 2000, Romalde et al. 2002). One strategy that has been developed for bivalve risk management is utilizing their capacity to eliminate harmful microorganisms and toxic substances when held in clean, disinfected seawater tanks for up to 72 h (Roderick & Schneider 1994, Wong et al. 1997, Sobsey & Jaykus 1999). A number of factors are believed to be important in establishing effective purification; like design, initial water quality, oxygenation and flow rates, salinity, temperature, shellfish to water ratios, removal and settlement of fecal material and the period of purification (De Mesquita et al. 1991, Lee & Younger 2002).

The development of the commercial fishery for the littoral burrowing clams *Ruditapes decussatus* and *Ruditapes philippinarum* are based mainly on the export of live products to Europe (Le Borgne 1996). The shelf life (survival and palatability) of this product depends on a number of factors including postharvest storage time and temperature. There are obvious advantages in being able to prolong shelf life without affecting the sensory quality adversely, and achievement of this requires an assessment of

the biochemical and physiological responses of the clams to anoxia/temperature combinations.

Total aquaculture production of bivalves in the world is 37,851,356 tons, this production value consist of 370,631 tons mussel, 4,207,818 tons oyster, 1,219,127 tons scallop and pectin and 3,109,024 tons clams. Total fisheries production of bivalves is 92,356,034 tons in the world. 257,315 of total production come from mussel, 199,015 tons come from oyster and scallop, 702,525 tons come from pectin and 808,945 tons come from clams (FAO, 2001).

Total fishery production of bivalves in Turkey is 624,847 tons, of which 5,000 tons come from mussel, 10,000 tons come from clam and 70 tons come from oyster. And also 2 tons of mussels come from aquaculture production (Anonymous 2002). The first exportation of the bivalves to Europe began in 1970 with clam *Ruditapes decussatus*. The most important species exported to foreign countries from Turkey are *Mytilus galloprovincialis*, *Ruditapes decussatus*, *Venus verrucosa*, *Ostrea edulis*, *Venus gallina* and *Arca* sp.

Several recent studies highlight the importance of using an integrated approach to assess environmental quality and effects of toxicants on organisms (Den Besten 1998, Livingstone et al. 1995, Mc Carthy & Shugart 1990). Differences in natural variables (i.e., sample site, exposure duration, population variation, temperature, salinity, etc.) complicate the identification of any biological responses to toxic substances. Transplantation of bivalve samples from a population at one location to other sites reduces the variability of results usually encountered in field sampling programs (De Kock & Kramer 1994, Amiard et al. 1987). Usero et al. (1997) studied trace metals in the bivalve mollusks *Ruditapes decussatus* and *Ruditapes philippinarum* from the Atlantic coast of southern Spain. Accumulation of metals, especially nonessential ones, is greatly dependent on their concentration in ambient water (Phillips & Rainbow 1989, Amiard et al. 1987) although there are other factors that affect accumulations such as the organism's size (Ringwood 1993, Brix & Lyngby 1985, Strong & Luoma 1981) and reproductive state (Paez-Osuna et al. 1995, Bordin et al. 1992). Concentrations of trace metals in *R. decussatus* and *R. philippinarum* reported in the study (Usero et al. 1997) were comparable to published data from less extensive surveys on the Southern Atlan-

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TABLE 1.  
Proximate composition of clams.

	Protein*	Fat*	Moisture*	Ash*	Carbohydrate*
<i>Ruditapes phillipinarum</i>	9.56 ± 0.06 <sup>a</sup>	0.98 ± 0.16 <sup>a</sup>	83.98 ± 0.16 <sup>a</sup>	3.3 ± 0.424 <sup>a</sup>	1.14 ± 0.02 <sup>a</sup>
<i>Ruditapes decussatus</i>	8.71 ± 0.18 <sup>b</sup>	0.78 ± 0.16 <sup>a</sup>	85.91 ± 1.22 <sup>a</sup>	3.1 ± 0.232 <sup>a</sup>	1.10 ± 0.02 <sup>a</sup>

\* Arithmetic means and standard deviation; Different superscripts between rows characterize significant differences ( $P < 0.05$ ). ( $n = 3$ ).

tic Coast of Spain (AMA 1992). Tuncer and Uysal (1982) found higher concentrations of Mn, Cr, Pb and similar concentrations for the other metals analyzed in *R. decussatus* in Izmir Bay (Turkey).

Bivalve mollusks have frequently been used as bioindicators for monitoring heavy-metal pollution because they are filter-feeding organisms with limited mobility (Storelli & Marcotrigiano 2001). The clams *Ruditapes decussatus* and *Ruditapes phillipinarum* were evaluated as possible biomonitors for Izmir bay and Venice bay. In this aim determining the differences of proximate and heavy metal contents is important.

#### MATERIALS AND METHODS

*R. decussatus* samples were taken alive from an aquaculture foundation, which was located in the province of Izmir city in Turkey, and the *R. phillipinarum* samples were taken alive from the fish market in Hamburg, in Germany. *Ruditapes phillipinarum* samples were imported from Venice, Italy. Analytical analysis of *Ruditapes decussatus* were performed in Turkey, on the other hand *Ruditapes phillipinarum* samples were analyzed in Federal Research center for fisheries, in Hamburg, Germany.

#### Biometric Measurements

Clams were immediately transported to the laboratory, where the fresh weights and linear dimensions (length, height and width) were determined. Clams were then opened, damp dried, soft tissues removed and shell and soft tissues weighed.

#### Proximate Composition

Proximate composition analysis of moisture (Ludorff & Meyer 1973), crude fat (Bligh & Dyer 1959) and crude protein (AOAC 1984) of *Ruditapes decussatus*, were performed and determined. Total volatile basic nitrogen (TVB-N) analysis was performed by using Kjeldhal Vapo 40 (Antonacopoulos & Vyncke 1989). Made in Turkey.

Proximate composition analysis of moisture (Oehlenschläger 1986), crude fat (Oehlenschläger 1986) and crude protein (Oehlenschläger 1997) of *Ruditapes phillipinarum* were performed and determined. TVB-N (Total Volatile Basic Nitrogen) analysis was performed by using Kjeldhal Vapo 50 (Rehbein et al. 1994). Made in Germany.

For proximate analyses and TVB-N analyses clam samples were pooled and homogenized. The analyses of the pooled samples were all carried out in triplicate.

#### Color Measurement

##### Instrument

The color measurement on samples trials was carried out with the spectral color meter Spectro-pen (Dr. Lange, Dusseldorf, Ger-

many). This is a colorimeter operating on the spectral method described in DIN 5033 using the 45/0° circular viewing geometry. The sample is illuminated with polychromatic light encircling it at an angle of 45°, with the optical unit observing the reflected light from a horizontal angle (0°) towards the sample surface. Spectro-pen is a genuine grating colorimeter measuring the visible spectral range (400–700 nm) at intervals of 10 nm. A 10° standard observers and D65 illuminant were used (light source: polychromatic with tungsten lamp). The PC software "spectral—QC" allows state-of-the-art data processing. Before measuring each lot, the colorimeter was calibrated against a white standard (LZM 224).

#### Measurement

The color measurement of clam samples trials was carried out with the spectral color meter Spectro-pen (Dr. Lange, Dusseldorf, Germany). The color was measured on homogenates prepared from each fish. The homogenate was placed in plastic petri dishes and the color measurement was repeated 10 times. In the CIE Laboratory system L\* denotes lightness on a 0–100 scale from black to white; a\*, (+) red or (–) green; and b\*, (+) yellow or (–) blue (Schubring & Meyer 2002).

#### Concentration of Trace Metals

Purpose of this study was to obtain quantitative information on the concentration of trace metals in two bivalve species from Izmir outer Bay and Venice, which samples were taken from the fish market in Hamburg, in Germany (Origin, Venice Italy).

The following points were considered: variations of metal concentrations among two species, variations of metal levels and the relationship between metal content and bivalve size. All samples were lyophilized in a FinnAqua Lyovac GT 2 freeze dryer (parameters: ambient temperature –20°C, vacuum 5–10 Pa, duration at least 48 h) and finally finely milled in a ball mill made from agate (Planetary Ball Mill, Fritsch, pulverisette 5, Idar-Oberstein, Germany). After milling, all samples were kept in high-density polyethylene bags at room temperature in an exsiccator until mineralization. Lyophilized samples (approximately 0.5 g) were weighed into petri dishes, which were put in a closed low-

TABLE 2.  
TVB-N & pH values

	TVB-N	pH
<i>Ruditapes phillipinarum</i>	1.55 ± 0.012 <sup>a</sup>	6.57 ± 0.01 <sup>a</sup>
<i>Ruditapes decussatus</i>	4.31 ± 0.015 <sup>b</sup>	6.52 ± 0.03 <sup>a</sup>

\* Arithmetic means and standard deviation; Different superscripts between rows characterize significant differences ( $P < 0.05$ ). ( $n = 3$ ).

TABLE 3.  
Biometrical measurements

	Total Weight* (g)	Length* (mm)	Width* (mm)	Flesh Weight* (g)	Shell Weight* (g)
<i>Ruditapes philippinarum</i>	13.66 ± 1.93 <sup>a</sup>	27.94 ± 1.61 <sup>a</sup>	39.33 ± 2.47 <sup>a</sup>	4.33 ± 1.04 <sup>a</sup>	8.34 ± 1.12 <sup>a</sup>
<i>Ruditapes decussatus</i>	11.46 ± 1.57 <sup>b</sup>	32.10 ± 3.01 <sup>b</sup>	33.67 ± 1.61 <sup>b</sup>	2.25 ± 0.43 <sup>b</sup>	8.88 ± 1.37 <sup>a</sup>

\* Arithmetic means and standard deviation; Different superscripts between rows characterize significant differences ( $P < 0.05$ ). ( $n = 10$ ).

temperature microwave activated oxygen plasma processor (Plasma Processor 200-G, Techniques Plasma, Munchen, Germany) for mineralization (power supply 350–360 W, vacuum 60–90 Pa, oxygen partial pressure 2.0–2.5\_105 Pa); the duration of decomposition was 144–168 h. The decomposed samples were quantitatively transferred into 100-ml volumetric flasks and dissolved in supra pure  $H_2SO_4$  (0.2%, w/w) at pH 2., DPSAV (746 VA Trace Analyzer, Metrohm, Switzerland) equipped with auto sampler (695 VA Auto sampler, Metrohm) and was used for the determination of heavy metals.

#### Statistical Analysis

SPSS 9.1 for windows program was used to search for significant differences between mean values of the different results. Differences between means were analyzed by 1-way analysis of variance (ANOVA) followed by Tukey and Duncan tests. Results are presented as means ± SD ( $n = 3$  or 4).

### RESULTS

The crude protein, total lipids, moisture, ash and carbohydrate contents of the clams are shown in Table 1.

According to their proximate composition data statistical difference occurred only in crude protein values. No statistical ( $P < 0.05$ ) difference was observed in the values of crude lipid, moisture, ash and carbohydrate. They were  $0.98 \pm 0.169\%$ ;  $83.98 \pm 0.16\%$ ;  $3.3 \pm 0.424\%$ ;  $1.14 \pm 0.02\%$ ;  $9.56 \pm 0.06\%$  for *Ruditapes decussatus*, respectively and  $0.78 \pm 0.16\%$ ;  $85.91 \pm 1.22\%$ ;  $3.1 \pm 0.232\%$ ;  $1.10 \pm 0.02\%$ ;  $8.71 \pm 0.18\%$ , for *Ruditapes philippinarum*, respectively. Protein content of *Ruditapes philippinarum* was higher than *Ruditapes decussatus*.

The TVB-N and pH values of the raw material are shown in Table 2.

In seafood, total volatile basic nitrogen (TVB-N) primarily includes trimethylamine (TMA), ammonia and dimethylamine (DMA). Each of these compounds, as well as total levels of TVB-

N, are useful indices of spoilage in different fresh and lightly preserved seafood. The European Commission (E.C. 1995) specified TVB-N to be used if sensory evaluation indicates doubt about freshness of different fish species. Critical limits of 25, 30 and 35 mg-TVb-N/100g were established for different groups of fish species. In processed, lightly or semi preserved, seafood levels of TVB-N at sensory product rejection are more variable. There was a significant difference between two data but this difference did not mean that those raw materials were not fresh. Total volatile basic nitrogen (TVB-N) includes all volatile amines. TVB-N is a spoilage index for fish and shellfish (FAO 1986). It is indicated that samples including lower than 25 mg N/100 g TVB-N value are in "perfect quality," samples including 30 mg N/100 g TVB-N value are in "good quality," samples including 35 mg N/100g TVB-N are in 'marketable quality' and the samples including more than 35 mg N/100 g TVB-n value are indicated as "spoiled" (Schormuller 1968, Ludorf & Meyer 1973). No statistical ( $P < 0.05$ ) difference was observed in the values of pH.

The biometrical measurements of the clams are shown in Table 3.

According to their biometrical measurements *Ruditapes philippinarum* had mostly higher values than *Ruditapes decussatus* except one criterion, which was the length. These results showed us that *R. philippinarum* is a little bit bigger than *R. decussatus*.

The color measurement data of the clams are shown in Table 4.

Their color data were very different as shown in the Table 4. *R. philippinarum* was much lighter, much redder and much yellower.

The two bivalves accumulated different amounts of metals in their tissues. Analysis of variance showed that the concentrations of zinc, cadmium, lead, copper and arsenic were significantly higher in *R. philippinarum* than in *R. decussatus*. Usero et al. (1997) found lower concentrations of zinc, cadmium, lead, copper and similar concentrations for the other metals analyzed in *R. decussatus* in Izmir Bay (Turkey).

Lead is considered to be a toxic element as a consequence of a variety of biochemical effects. These include neurological problems, hematological effects, renal dysfunction, hypertension and cancer, for which there is evidence for animals but not yet for humans. The maximum heavy metal levels permitted in Turkey are 0.1 mg/kg for cadmium and 1.0 mg/kg for lead according to the Turkish Food Codex (1997). So all the values are reasonable for human life according to Turkish Food codex.

### CONCLUSION

In this study proximate composition and biometrical size of two clams was compared. These clams were all of commercial value in the seafood industry. The aim of this study was determining the differences of these two species. Although there are some differ-

TABLE 4.  
Color measurement

	L*	a*	b*
<i>Ruditapes decussatus</i>	36.21 ± 3.67 <sup>a</sup>	-0.50 ± 0.14 <sup>a</sup>	10.94 ± 1.158 <sup>a</sup>
<i>Ruditapes philippinarum</i>	70.53 ± 3.12 <sup>b</sup>	4.11 ± 0.80 <sup>b</sup>	23.51 ± 1.00 <sup>b</sup>

\* Arithmetic means and standard deviation; Different superscripts between rows characterize significant differences ( $P < 0.05$ ). The color was measured on homogenates prepared from each clam. L\* (lightness), a\* (redness), b\* (yellowness). ( $n = 10$ ).

TABLE 5.  
Concentrations of Zinc, Cadmium, Lead and Copper

	Zinc (mg/kg)	Cadmium (µg/kg)	Lead (µg/kg)	Copper (mg/kg)
<i>Ruditapes phillipinarum</i>	110.8 ± 12.02	562.55 ± 2.47	376.05 ± 3.88	18.95 ± 0.77
<i>Ruditapes decussatus</i>	22 ± 17	250 ± 006	370 ± 0.05	3.4 ± 0.36

(Mean ± standard deviation)

ences, these differences do not mean that the Turkish clam would not be alternative specie for the seafood industry. And also according to their trace metal contents all the values are under the limits of consumption according to Turkish food codex. Although

fishery activity and the culture of this species has some difficulties, with the support of new scientific investigations and the commercial support of the foundations, this clam could become a real alternative choice to the European species on an industrial scale.

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## BIOTIC AND ABIOTIC FACTORS INFLUENCING GROWTH AND SURVIVAL OF WILD AND CULTURED INDIVIDUALS OF THE SOFTSHELL CLAM (*MYA ARENARIA* L.) IN EASTERN MAINE

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**ABSTRACT** A series of intertidal field experiments was conducted from 1986–2003 in eastern Maine to examine biotic and abiotic factors influencing the growth and survival of wild and cultured individuals of the softshell clam, *Mya arenaria* L. Separate experiments examined: (1) the efficacy of transferring sublegal wild clams (<50.8 mm SL) from areas near the high intertidal zone where shell growth was slow to areas where growth was predicted to be faster; (2) effects of tidal height on wild and cultured clam growth; (3) effects of spatial variation on cultured clam growth; (4) dispersion and growth of cultured juveniles in small experimental units; (5) effects of the naticid gastropod, *Euspira heros* Say, predation on survival of wild and cultured clams and (6) the species composition of large, crustacean predators that forage intertidally during periods of tidal inundation. Protective netting (4.2 mm aperture) increased recovery rate of transferred clams by 120% and resulted in a 3-fold enhancement of wild recruits. Effects of tidal height on wild clam growth revealed complex behaviors in >0 y-class individuals. Clams growing near the upper intertidal take >8 y to attain a legal size of 50.8 mm SL, whereas animals near the mid intertidal generally take 4.5–6.5 y. Unexpectedly, clams initially 38–54 mm SL and growing near the extreme low tide mark at a mud flat in Eastport, Maine, added, on average, <2 mm of new shell in a year, which was 8–10 mm SL less than animals at higher shore levels. It is hypothesized that biological disturbance by moon snails, that consumed >90% of clams at the low shore levels, contributed to this slow growth. In another field trial from 1986–1987, moon snails and other consumers were allowed access to clams ranging in size from 15–51 mm. *E. heros* preyed on clams over the entire size range and attacked clams between 31–40 mm at a rate that was nearly double what had been expected. Mean snail size was estimated to range from 10–52 mm shell height (SH), based on a laboratory study that yielded information about the linear relationship between snail size and its borehole diameter. In an experiment from June to September 1993, moon snails consumed >70% of juvenile clams (ca. 10 mm SL) within a month after planting at each of three tidal heights. Snail sizes ranged from 15–20 mm SD with larger individuals occurring near the upper intertidal zone. Green crabs, *Carcinus maenas* (L.) also prey heavily on softshell clam populations, but most studies that use shell damage to assign a predator have assumed that all crushing and chipping predation is because of this invasive species. An intertidal trapping study demonstrated that both green crabs and rock crabs, *Cancer irroratus* Say, are present during periods of tidal inundation, with the latter species accounting for ca. 40% of large crustacean numbers.

**KEY WORDS:** *Mya arenaria*, softshell clam, Maine, growth, predation, *Euspira heros*, *Carcinus maenas*

### INTRODUCTION

In Maine, USA, softshell clams, *Mya arenaria* L., are the third most important commercial marine species harvested behind lobster, *Homarus americanus* Milne Edwards, and cultured salmon, *Salmo salar* L. From 2001–2004, dockside clam landings in Maine averaged ( $\pm 95\%$  CI)  $1113.9 \pm 99.7$  metric tons (worth \$15.9  $\pm$  \$1.68 million; Fig. 1), worth more than \$50 million annually to the state economy (ME DMR 2005).

Each of Maine's 105 coastal communities has the option to manage its intertidal clam stocks within its municipality using one of the oldest comanagement programs in the United States. Beginning in 1962, Maine's State Legislature created a community-based management structure between municipalities and the State of Maine through the Department of Marine Resources (DMR). This structure, or agreement, is called a "shellfish ordinance," and it allows towns to adopt, amend or repeal a set of shellfish conservation measures that regulate the taking of shellfish within the intertidal zone of the municipality. The ordinance is a broadly defined document that includes language about conservation activities, qualifications of a licensee, license fees, limiting effort, harvesting methods and tools, enforcement, the organization and rights of a local stewardship committee and other measures. The first step a town must take once it declares its intention to manage its local clam assets is to adopt a "model ordinance," or general management plan, that outlines basic requirements the state man-

dates. (Towns not interested in managing their clam stocks are not required to do so, and, therefore, the State must act alone to manage those intertidal flats to the best of its ability.) However, the model ordinance is structured to permit sufficient flexibility to enable a community to benefit from local knowledge, wants and wishes by creating, adopting and regulating its unique management schemes that may exceed the basic provisions set out in the model ordinance. These local modifications are enacted when a community requests them in writing to the DMR, and they are granted by the DMR Commissioner. In this way, two adjacent communities may have a common goal of increasing local clam stocks, but have very different looking shellfish ordinances. There are 3 common requirements of all communities with shellfish ordinances: (1) the management plan be enforced by a warden paid through the town; (2) no clam less than two-inches (50.8 mm) in shell length (SL) can be harvested legally and (3) 10% of licenses sold in a community must be made available to Maine citizens outside the particular municipality. In 2005, 73 communities (or 70%) with intertidal habitat chose to manage those areas for *M. arenaria* production.

Since the inception of the shellfish ordinance, local stewardship committees have adopted a number of shellfish management tools in an attempt to maintain high yields of clams and keep their clamming habitat productive. The following list includes management activities that have been used in Maine since 1962—no community has adopted all of the activities: (1) limiting the number of licenses sold; (2) restricting harvest volumes; (3) limiting all harvesting to recreational diggers; (4) limiting when harvesting can

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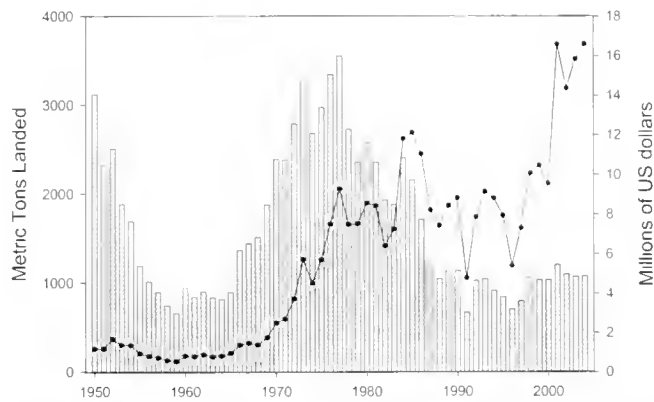


Figure 1. Commercial landings of soft-shell clams in Maine from 1950 to 2004. Data from: <http://www.maine.gov/dmr/commercialfishing/softshellclam.htm>.

occur (e.g., no Sunday digging; no night digging); (5) restricting the areas where harvesting can occur (i.e., flat rotations); (6) applying tree brush or snow-fencing to intertidal areas to encourage juvenile clams settlement; (7) applying wire fencing or plastic netting to deter green crab predation and encourage juvenile clam settlement; (8) assessing stock volume and size frequency distributions (i.e., clam surveys); (9) transferring clams from high density/slow growth areas to low density/fast growth areas; (10) enhancing flats with hatchery-reared, or cultured clam seed; (11) installing municipal sewage treatment systems and reducing over-board discharges (e.g., water quality monitoring programs) and (12) municipal leasing of flats (each community has the right to set aside up to 25% of its productive clamming habitat for private clam farming operations).

Shellfish committee members, in concert with State resource managers, must make decisions concerning the status and health of soft-shell clam populations for a variety of applications, the most common being whether the population is abundant enough to be harvested in a sustainable fashion and what level of harvesting will minimize impacts on future populations. Because of logistical constraints imposed by working in marine environments, managers of marine resources often have limited information about important population characteristics such as survival, growth and recruitment rate and how these parameters change spatially and temporally. Rather, decisions about harvest levels, for example, usually are limited to estimates of change in standing stocks and size frequencies through time or between locations.

It is rare that adaptive management strategies and experimental approaches are considered by fisheries managers (but see Botsford et al. 1997, Lenihan & Micheli 2000, Beal & Vencile 2001); however, manipulative field experiments are the strongest and most efficient means available to managers to base decisions about the dynamics of a population (Underwood 1990, Underwood 1991). The field trials reported here are intended to add to a growing literature on soft-shell clam ecology from Maine and western New Brunswick, Canada (Spear & Glude 1957, Welch 1969, Comito 1982a, Comito 1982b, Newell & Hidu 1982, Ambrose et al. 1998, Beal et al. 2001, Beal and Kraus 2002, Whitlow et al. 2003, Auffrey et al. 2004, Logan 2005) and provide information to shellfish stewardship committees and resource managers that increases the scope of their management toolbox.

This contribution summarizes a number of field investigations conducted in eastern Maine since 1986 that focuses specifically on

the biotic and abiotic factors affecting growth and survival of wild and cultured soft-shell clams.

## METHODS

### Experiment 1

#### Clam Transfers

In 2004, approximately 44% of the 73 Maine communities that actively managed their shellfish stocks participated in some form of transfer program (ME DMR 2005). These activities are very labor intensive because they involve harvesting and transporting wild "seed clams" from areas where growth is slow or retarded (i.e., near the upper intertidal where clam growth can be 95% slower than growth of the similar animals at the low intertidal [Beal et al., 2001]) to areas where growth is faster. Most communities transfer clam seed that is approximately 38–45 mm SL (pers. obs.). To the best of my knowledge, there has been only one attempt to quantify the efficacy of transfers in Maine. Here, information is presented that examines short-term survival, growth and recruitment patterns of transferred clams.

On May 2, 1998, approximately 165 kg of soft-shell "seed" clams were dug by commercial harvesters from the city of Eastport, Maine in the high intertidal zone of a flat near Carlow Island in Passamaquoddy Bay (44°56.34'N; 67°01.77'W). Animals were slow growing, and annual ring counts (Newcombe 1935) indicated that the oldest animals were >15 y old. Clams were sieved, washed, and stored overnight in a walk-in cooler at 4°C. A random sample of these clams ( $n = 96$ ) demonstrated that the ranges of SL's varied from 23.2 mm to 58.5 mm with a mean SL =  $39.3 \pm 9.5$  mm. A relationship between clam mass and clam number was developed for those clams by counting five separate lots of individuals at 0.45, 1.36, 2.27 and 3.18 kg (Count =  $0.03 + 107.21 \times \text{Mass}$ ;  $n = 20$ ;  $r^2 = 0.969$ ;  $P < 0.001$ ). This relationship was used to estimate number of clams seeded into plots (9.3 m<sup>2</sup>) at a nearby intertidal flat, Carrying Place Cove, Eastport, Maine (44°54.04'N; 67°01.28'W), at low tide on 3 May 1998. Seeding densities were 0, 4.1, 6.1 and 12.3 kg per plot, or 0, 440, 654 and 1319 clams per plot representing approximately 0, 47, 70 and 142 animals m<sup>-2</sup>.

Six treatments, with five replicate plots each, were established: (1) 0 kg with a piece of 4.2 mm flexible plastic netting to deter predators; (2) 4.1 kg with netting; (3) 4.1 kg with no netting and surface of flat roughened with clam harvesting hoes (*sensu* Robinson & Rowell 1990); (4) 6.1 kg with no netting; (5) 6.1 kg with 4.2 mm flexible plastic netting and (6) 12.3 kg with no netting. Treatments were randomly assigned to plots that were established in a 6 × 5 matrix with 5 m between rows and columns. An initial survey of the experimental area failed to locate a single living clam. To estimate the short-term effect of enhancing flats through transferring seed clams, each plot was randomly sampled twice on October 27, 1998 using a coring device (0.02 m<sup>2</sup>) to a depth of 20 cm. All transferred clams per core sample were counted and the SL of each live clam was measured to the nearest 0.1 mm using Vernier calipers. It was possible to distinguish "new shell growth" as an obvious white band along the entire ventral margin of the clam.

Several *a priori* contrasts were of interest for both growth and survival variables: (1) Is netting important to retain transferred clams? This was tested by comparing treatments 4 & 5; (2) Does roughening the sediment surface enhance the burrowing rate of clams? This was tested by comparing treatments 2 & 3 and (3) Is

there an effect caused by seeding density? This was tested by comparing the mean of treatments 2 & 4 versus 6. In addition, the control plots (without seed) were used to compare recruitment of wild spat (animals <18 mm SL) in netted plots to similar plots containing transferred seed. This was a comparison of the mean of treatment 1 versus the mean of treatments 2 & 5. A conservative decision rule was used for each contrast based on advice from Winer et al. (1991) who cautioned against excessive type I error by reducing  $\alpha$  using the following equation:

$$\alpha' = (1 - \alpha)^{1/m}$$

where  $m$  = the number of contrasts.

In this instance, because  $m = 3$ ,  $\alpha'$  equals 0.0170.

## RESULTS

### Survival

Between 30% and 70% of clams initially transferred were missing from plots at the end of the 5-mo experimental period. Greatest missing rates occurred in the low-density plots. Table 1 demonstrates that protecting clams with netting had no statistically significant effect on clam density after 5 mo ( $P = 0.0293$ ); however, mean density under the netting was approximately twice that in plots without netting ( $220 \pm 35.9$  vs.  $100.0 \pm 36.5$  animals  $m^{-2}$ ;  $n = 5$ ). There was no significant difference in final density between roughened versus nonroughened plots ( $20.0 \pm 14.5$  vs.  $15 \pm 6.0$  individuals  $m^{-2}$ , respectively). In addition, significant differences in final clam density were observed after 5 mo; however, instead of a 3-fold difference between highest and smallest density treatments that was incorporated into the experimental design (see earlier), a 5-fold difference in final density was observed ( $95.0 \pm 24.0$  vs.  $17.5 \pm 10.3$  animals  $m^{-2}$ ; Table 1;  $P = 0.0074$ ).

### Growth

Clam growth was related to initial planting size (Fig. 2), but was unaffected by stocking density, netting and sediment roughening ( $P > 0.80$ ). Clams initially <35 mm added, on average,  $8.1 \pm 1.14$  mm ( $n = 13$ ) of new shell between May and October 1998 compared with  $3.4 \pm 0.56$  mm ( $n = 27$ ) for animals >35 mm ( $P = 0.0002$ ).

TABLE 1.

Analysis of variance on the square root-transformed number of re-seeded clams per 0.02  $m^2$  sample taken on October 27, 1998 at Carrying Place Cove, Eastport, Maine. Two random samples were taken from each of five replicates of each treatment ( $2 \times 5 \times 5 = 50$  samples). A priori contrasts appear under the Treatment source of variation and a decision rule equal to  $\alpha' = 0.0170$  was used.

Source of Variation	df	SS	MS	F	Pr > F
Treatment	4	10.84	2.71	11.87	0.0091
(1) Net versus no net	1	1.54	1.54	8.11	0.0293
(2) Roughened versus not	1	0.01	0.01	0.02	0.8952
(3) Stocking density	2	4.58	2.29	37.84	0.0074
Sample (Treatment)	5	1.14	0.23	0.59	0.7071
Error	40	15.46	0.39		
Total	49	27.44			

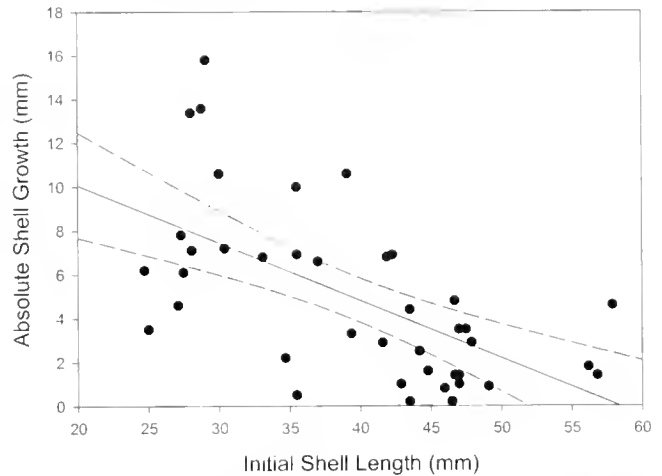


Figure 2. Absolute growth ( $\pm 95\%$  confidence interval) of softshell clams transferred to Carrying Place Cove in Eastport, Maine from May 2 to October 27, 1998.  $Y = 15.3 - 0.262 X$ ;  $n = 40$ ;  $r^2 = 0.368$ ;  $P < 0.001$ .

### Recruitment

No wild spat were found in control plots that did not receive clam seed, nor were spat found in samples from the low density stocking treatment. ANOVA on the square root-transformed number of wild spat (<18 mm SL) per core (Table 2) demonstrated a significant effect because of the presence of the netting as 3.3 times as many spat were found in netted versus unnetted plots ( $50.0 \pm 21.1$  versus  $15.0 \pm 7.6$  individuals  $m^{-2}$ ;  $n = 5$ ). In addition, number of wild spat increased significantly with stocking density ( $P = 0.0087$ ; Table 2) from  $15 \pm 7.2$ – $45 \pm 10.3$  individuals  $m^{-2}$  ( $n = 5$ ) in plots initially seeded at densities of 70, and 142 animals  $m^{-2}$ , respectively. No differences in SL were observed across the seeding treatments for the wild spat ( $P = 0.8606$ ).

### Experiment II

#### Growth of Soft-shell Clams in Eastern Maine

Clam growth rates vary geographically along the coast of Maine. Dow and Wallace (1953) reported that in some areas of eastern Maine, it takes an average of 8 y for *Mya* to reach legal size

TABLE 2.

Analysis of variance on the square root-transformed number of wild clam spat (0-year class individuals <18 mm SL) per 0.02  $m^2$  sample taken on October 27, 1998 at Carrying Place Cove, Eastport, Maine.

Two random samples were taken from each of five replicates of each treatment ( $2 \times 6 \times 5 = 60$  samples). A priori contrasts appear under the Treatment source of variation and a decision rule equal to  $\alpha' = 0.0170$  was used.

Source of Variation	df	SS	MS	F	Pr > F
Treatment	5	5.95	1.19	13.81	0.0031
(1) Net versus no net	1	0.90	0.90	10.44	0.0169
(2) Roughened versus not	1	0.00	0.00	0.00	1.0000
(3) Stocking density	2	2.65	1.32	33.91	0.0087
Sample (Treatment)	6	0.52	0.09	0.39	0.8834
Error	48	10.67	0.22		
Total	59	17.14			

(50.8 mm SL). This average decreases in a southwesterly direction where, in the area from Portland to Kittery, it takes approximately 3 y for animals to attain legal size. In addition, tidal inundation may influence growth rates as clams cease growing during periods when the tide leaves the mudflats. To investigate how clam growth is influenced by tidal position, a number of studies have been carried out during the past decade in eastern Maine. Here, results from four investigations are presented that were conducted in Eastport, Jonesport, and Addison.

## METHODS

### Eastport

Twenty 0.25 m<sup>2</sup> plots (corners marked with wooden stakes) were established at Carrying Place Cove on April 22, 1998 at 10 separate locations ranging from extreme low tide (plots 1–3) to lower mid tide (plots 4–6) to upper mid tide (7–9) to upper tide (plot 10). At each location, replicate plots were established. Wild clams used in the study were dug on April 19, 1998 near Carlow Island (described in the **Clam Transfers** section) and ranged from 23.8–59.0 mm SL. Animals were held in a cold room at 4°C, and on April 21, approximately 170 clams were uniquely marked with an oil-based ink (Mark-Tex Corp.) and measured to the nearest 0.1 mm using Vernier calipers. To facilitate planting the clams and to aid in their burrowing, on April 22 the surface of each plot was roughened using a clam hoe. Then, 17 marked and 10 unmarked clams were pushed into the sediments (so that the posterior margin of each clam was 1 cm below the sediment) in one of the two plots (a) at each location 27 unmarked individuals similarly were planted in the other plot (b). Sediment type varied from location to location along the tidal gradient. Plots became sandier towards the mid-tide area (except for plot 7, which was located in a blue clay sediment). Plots 1–3 typically were exposed only briefly (ca. 30–40 min) on the spring tides of each month, and clam growth rate in these plots was predicted to be the fastest because these animals were able to feed longer each tidal cycle than clams in plots at the higher tidal levels. Conversely, growth rate of clams in plots at the uppermost tidal level was predicted to be the slowest because those plots were exposed daily for the most amount of time during each semidiurnal tidal cycle (ca. 240–300 min).

On April 20, 1999, all 20 plots were excavated and all live and dead animals were taken to the laboratory. Each live clam was examined for a disturbance line believed to coincide with the size of the clam on 22 April 1998 (see an example of this technique for juveniles of *M. arenaria* in Beal et al. 1999). The marked clams enabled me to test the hypothesis that the disturbance line coincided with the size of the animal when it was transferred to the 0.25 m<sup>2</sup> plot. Using calipers, the length of the marked animal at the disturbance line was estimated and recorded. This size is referred to as a "predicted length." After all marked animals from each plot had been measured, these predicted lengths were compared with the actual lengths that had been recorded the previous year. A perfect correlation would result in a line with the equation  $Y = X$ . A 2-tailed, one-sample *t*-test was performed to test whether the difference between actual and predicted was zero. Fifty-nine marked individuals (of 170) were found alive in all 10 locations. The mean difference between the actual length and the predicted length for these 59 clams was 0.098 mm (minimum difference was –0.5 mm and the maximum difference was 1.4 mm; Fig. 3). The test revealed that the mean difference was not significantly different from zero ( $T = 1.836$ ;  $df = 58$ ;  $P = 0.072$ ), suggesting that

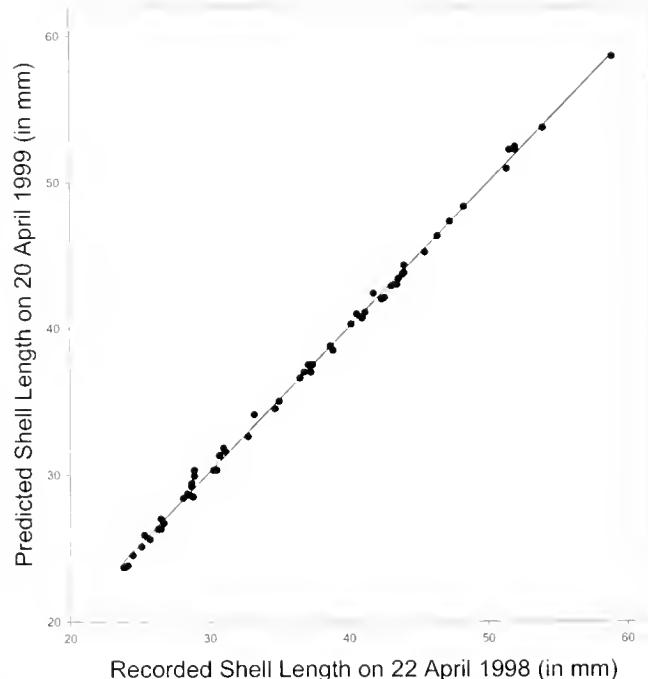


Figure 3. Predicted versus recorded shell length of marked clams from Carrying Place Cove, Eastport, Maine, on April 20, 1999. Predicted SL was based on an obvious disturbance mark in the valve of clams that had been marked with ink near their umbo on April 22, 1998. Because the equation of the line ( $Y = -0.55 + 1.012 X$ ;  $n = 40$ ;  $r^2 = 0.998$ ) is not significantly different from  $Y = X$ , the disturbance line was used to establish the size of unmarked clams in the growth rate experiment.

the disturbance line on each valve coincided directly with the handling, planting and other disturbance the animals had endured a year earlier. Because clam growth over the entire year could be discerned, Ford-Walford plots (Walford 1946) were used and then converted to von Bertalanffy equations. These equations were plotted to generate plot- and tide-specific growth rate curves.

### Jonesport

Hatchery-reared clams (12.4 mm  $\pm$  0.31 mm), produced in 1995 at the Beals Island Regional Shellfish Hatchery (BIRSH; Perio Point, Beals, Maine) and overwintered according to Beal et al. (1994), were placed into plastic horticultural plant pots (15 cm diameter  $\times$  15 cm deep) that had been filled with ambient sediments at an intertidal flat (Flake Point Bar, 44°36.75'N; 67°33.72'W) near Jonesport, Maine on 6 April 1996. Experimental units were distributed in three locations along a shore level gradient (low, mid and high intertidal) and stocked at one of three intraspecific densities (330, 660 and 1320 m<sup>-2</sup>). One-half of all units were covered with a piece of flexible, plastic netting (6.4 mm aperture) to deter predators (Beal et al. 2001). Ten replicates of each of the six treatments were used at each tidal height. To assess clam growth rate, units were collected on December 13, 1996, approximately one month after shell growth ceases in this region (Beal 1994) and two linear measurements (initial and final SL) were taken on all live clams using Vernier calipers to the nearest 0.1 mm. It was possible to discern the initial size of each hatchery-reared clam even though none was marked uniquely because each animal lays down a unique disturbance check in both of its valves at the time it is placed in the sediments (Beal et al. 1999).

In another study at Flake Point Bar, wild clams (size range = 21.4–79.5 mm) were collected from the upper ( $n = 17$ ) and mid ( $n = 23$ ) intertidal on November 11, 2004, and the animals aged using annual rings (Newcombe 1935) to estimate tide-specific age-length data. Ford-Walford plots were used to generate von Bertalanffy growth curves.

#### Addison

On May 10, 2001, hatchery-reared clams ( $12.4 \pm 1.4$  mm SL; stocking density = 535 individuals  $m^{-2}$ ) were added to 24 plastic horticultural pots (as described earlier) that contained ambient sediments at 4 intertidal sites chosen by Addison's shellfish committee (Batson's Beach:  $44^{\circ}31.65'N$ ;  $67^{\circ}41.98'W$ , Eastern Harbor:  $44^{\circ}30.93'N$ ;  $67^{\circ}43.37'W$ , Three Brooks:  $44^{\circ}33.56'N$ ;  $67^{\circ}44.81'W$ , Upper Pleasant River:  $44^{\circ}35.82'N$ ;  $67^{\circ}44.58'W$ ). At each site, experimental units were arrayed in six blocks of four units each ( $2 \times 2$  matrix per block with 1-m spacing between rows and columns). To deter predators, in each block, a thin, flexible piece of plastic mesh netting (aperture = 6.4 mm) was affixed to the top of two units using rubber bands, whereas the other two received no netting. Blocks were spaced about 2 m apart and placed between the mid to low intertidal at each site. All units were removed from three of the sites on November 16, 2001, whereas the units were removed from Three Brooks on November 21, 2001. Growth rates for each living clam was determined as described earlier.

ANOVA was conducted on the untransformed mean final SL using the following linear model:

$$Y_{ijkl} = \mu + A_i + B_j + AB_{ij} + C(A)_{k(i)} + BC(A)_{j(k)} + e_{l(ijk)},$$

where

$\mu$  = theoretical mean;

$A_i$  = site ( $i = 4$  sites, factor is fixed);

$B_j$  = Netting Treatment ( $j = 2$ , protected versus unprotected, factor is fixed);

$C_k$  = Block ( $k = 6$  blocks per site; factor is random); and,

$e_l$  = experimental error.

## RESULTS

### Eastport

Most (93%) of the clams in the lower plots were recovered dead with a countersunk hole, typical of predation by the moon snail, *Euspira heros* Say. Only 1 of 68 marked clams was found alive in plots 1a–4a (lowest tidal heights). That animal grew 1.5 mm over the year from 49.6–51.1 mm. Similarly, in a plot with unmarked animals (3b), one live clam (of 27 planted) was found, and it had grown only 4 mm from 38.0–42.0 mm. Plot 4b contained only 3 live, unmarked clams (one had grown 1.9 mm from 54.1 mm to 56.0 mm, the second grew 0.5 mm from 49.1 mm to 50.6 mm, and the third grew 0.8 mm from 48.5 mm to 49.3 mm).

Clam survival in lower mid to upper intertidal plots at Carrying Place Cove (5–10) varied from 50% to 81%, which yielded sufficient data to estimate annual growth rates (Fig. 4). The time to reach a legal size of 50.8 mm SL varied directly with tidal height and sediment type. The growth curves of clams from plots near the lower mid tide (Plots 5 & 6) demonstrate that it would take between 4.5–5.5 y to reach 50.8 mm. Clams in clay sediments within

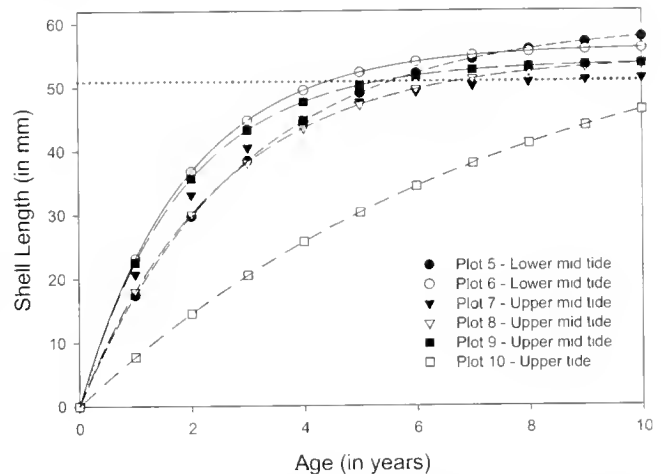


Figure 4. von Bertalanffy growth curves of wild clams along a tidal gradient at Carrying Place Cove, Eastport, Maine from April 22, 1998 to April 20, 1999. The dotted line parallel to the x-axis is the minimum legal size of softshell clams in Maine (50.8 mm, or 2-inches SL).

Plots 7a, b, near the upper mid tide, take 7–8 y to reach legal size; however, animals in adjacent plots at the same tidal height but in more sandy sediments (Plots 8a, b) reach this size in 6.5 y. Growth rate of clams at or near the highest tidal mark are extremely slow (Fig. 4), and animals there may never attain legal size (pers. obs.).

### Jonesport

No effect caused by predator exclusion ( $P = 0.0696$ ) or stocking density ( $P = 0.0890$ ) on clam growth was detected from April to December 1996; however, tidal height effects were highly significant ( $P < 0.0001$ ). Final mean SL of animals at the lowest intertidal was 35% greater than those at the mid tide level and 95% greater than those at the upper intertidal. In addition, growth variation decreased significantly from the lowest to highest tidemark (Fig. 5).

From the November 2004 sampling, clam growth was faster at the mid intertidal versus the upper intertidal reflecting differences in tidal inundation (Fig. 6). Clams attain a size of 50.8 mm in 5.8 y at the mid-tide level and 7.8 y near the upper intertidal.

### Addison

No differences in growth were observed between clams protected with netting and those that remained unprotected at any of the four sites for the 198-day experiment. Growth rate was fastest at Eastern Harbor where clams increased in SL by an average of 27.0 mm (final mean SL =  $39.4 \pm 1.2$  mm) and slowest at Upper Pleasant River where final mean SL was  $31.2 \pm 3.5$  mm. A small percentage of clams (1.9% and 1.0%) attained at least 50.8 mm SL at Eastern Harbor and Three Brooks, respectively (Fig. 7). ANOVA indicated that site was the only significant source of variation (Table 3) and an *a posteriori* Student-Newman-Keuls (SNK) test demonstrated that clams at Eastern Harbor and Three Brooks attained significantly greater final mean SL's than clams at the other two sites ( $P = 0.0001$ ).

### Experiment III a and b

#### Dispersion and Growth of Hatchery-reared Juveniles of the Softshell Clam

Many of the small-scale field experiments conducted since 1989 (Beal 1994, Beal et al. 2001; Beal & Kraus 2002) have used

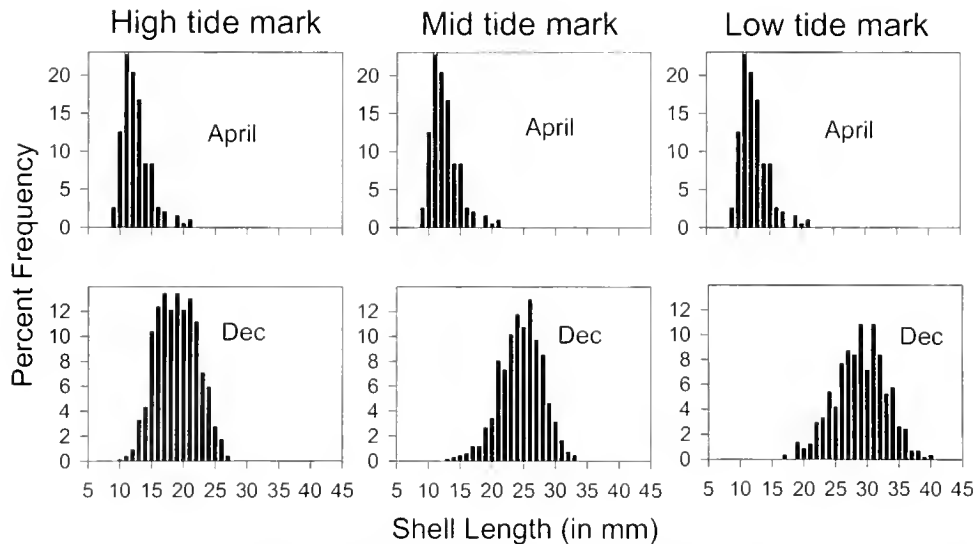


Figure 5. Size-frequency distributions of hatchery-reared clams planted at three different tidal heights at an intertidal flat near Jonesport, Maine from April 6 to December 13 1996. Mean initial SL = 12.4 mm. Most shell growth occurred between early June and August 6, but rates depended on tidal height (high, mid, and low tide plots = 55%, 42.2% and 45.2%, respectively).  $n_{\text{April}} = 191$  for all tidal heights;  $n_{\text{December}}$  for high tide, mid tide and low tide clams = 758, 670 and 571, respectively.

plastic, horticultural plant pots (15.2 cm diameter  $\times$  15.2 cm deep with a cross sectional area of 0.0182 m<sup>2</sup>). These experimental units are filled with ambient, unsieved sediments, and then buried to their rims in the sediments of a particular intertidal mud flat. Clam juveniles are then pushed gently to a depth of 12–15 mm under the sediment surface within the experimental units. These units are relatively easy to manipulate and establish various treatments within (e.g., stocking density, initial clam size, etc.). To discourage clams from emigrating from these units, a strip of flexible, plastic netting (4.2 mm aperture and measuring 10 cm  $\times$  50 cm) typically is affixed to the outside circumference of each pot creating an open enclosure (*sensu* Beal et al. 2001). The netting, which extends 4–5

cm above the lip margin, purportedly acts as a fence to further decrease the probability of animals somehow escaping by themselves. Strips of the netting do not cover the top of the experimental unit and, therefore, allow predator access to the clams within the experimental units. Often, results of short-term field experiments reveal that missing rates from these open enclosures can be as high as 30% to 40% (Beal et al. 2001). Because the strip of netting is supposed to act as a barrier to emigration, it has been assumed that the missing clams are dead, the result of one or more predation events that directly or indirectly (tidal currents, wind, etc.) remove animals or their shells from the experimental units. In addition, it has been assumed that the strips of netting do not modify local currents and result either in a reduction or enhancement of shell growth.

## METHODS

### Experiment III a

To test the assumption that the flexible netting strips affixed to the experimental horticultural pot enclosures provide a barrier to migration, a laboratory and field test was designed. In the laboratory at BIRSH, 10 experimental units containing muddy sediments obtained from a nearby mudflat were established (as described earlier). On April 5, 1989, twelve hatchery-reared clams (4–6 mm SL) were added to each enclosure, and then the experimental units were placed in an array on the bottom of a 2,600-L fiberglass tank filled with ambient, unfiltered seawater (4°C). After one hour, during which time all clams had burrowed completely into the sediments, seawater was permitted to flow into the tank at a rate of ca. 5 l min<sup>-1</sup>. After seven days at this flow rate, sediments in each experimental unit were washed through a 0.5-mm sieve, and all living and dead clams enumerated.

Emigration may depend on flow rate, varying submergence times, changes in temperature between immersion and emersion and other variables that are not controlled in a field setting. It is possible that experimental units with strips of mesh netting surrounding the periphery will act like a predator inclusion cage,

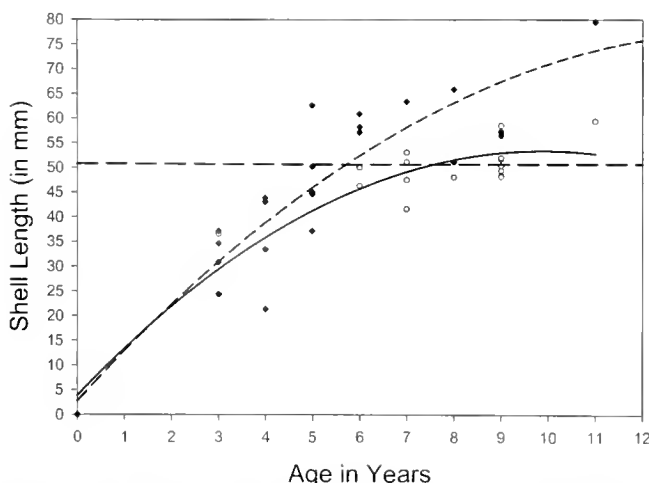


Figure 6. Age-length curves for wild clams collected on November 11, 2004 from two tidal heights at an intertidal flat near Jonesport, Maine. The dashed curve (diamond symbols) represents growth of clams near the mid intertidal ( $n = 17$ ) whereas the solid curve (open circles) represents growth of clams near the upper intertidal ( $n = 22$ ). The dashed line parallel to the x-axis is 50.8 mm, or minimum legal size (2-inches SL).



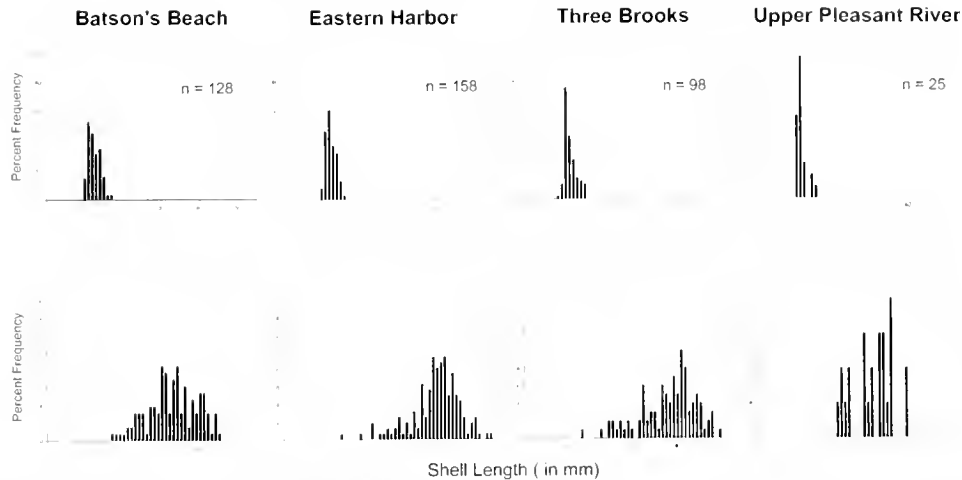


Figure 7. Size-frequency distribution of hatchery-reared softshell clams planted between the mid and low tide mark at four intertidal flats near Addison, Maine (May 10 to November 21 2001). Fastest growth occurred at Eastern Harbor and Three Brooks, where final mean SL  $\pm$  95% CI =  $38.9 \pm 0.98$  mm ( $n = 38$ ) was significantly different ( $P < 0.05$ ) from the final mean SL at the other two sites ( $32.8 \pm 1.41$  mm;  $n = 31$ ).

enhancing predation by crabs and other consumers. Conversely, the flexible netting strip may deter predators and keep predation rates artificially low. Because laboratory studies cannot adequately reflect field conditions, the potentially confounding effects of emigration from experimental units also were evaluated in the field near the mid intertidal at a mudflat in the town of Cutler, Maine ( $44^{\circ}41.22'N$ ;  $67^{\circ}18.59'W$ ). On May 22, 2003, cultured clams (mean SL =  $11.7 \pm 0.25$  mm) were added to experimental units (plastic horticultural pots) with and without a strip of flexible netting (as described earlier) at two stocking densities (12 or 24 clams representing  $660$  and  $1320$   $m^{-2}$ , respectively). One half of all units were surrounded by an open ring of solid plastic (11.5 cm wide  $\times$  29.0 cm diameter) that was pushed into the sediments 65 mm so that the experimental unit was in the middle of the open ring. The ring was used to help further contain clams that might emigrate from experimental units. The experiment was established as a completely randomized design with 10 replicates for each of eight fully factorial treatments (stocking density,  $a = 2$  levels; netting strip,  $b = 2$  levels; plastic ring,  $c = 2$  levels). Enclosures were added to a  $10 \times 8$  matrix, with 1-m spacing between rows and columns. Both short- and longer-term treatment effects were stud-

ied by randomly removing five replicates from each treatment from the matrix after 8 days, on May 30, 2003 and the remaining five replicates after 100 days, on August 30, 2003. Sediments from each experimental unit were washed through a 2-mm mesh. In addition, for units surrounded by a plastic ring, sediments within the area of the ring minus the area occupied by the experimental unit (i.e.,  $0.066$   $m^2 - 0.018$   $m^2 = 0.048$   $m^2$ ) were sampled to a depth of 14 cm. Each of these samples was processed as described earlier. The relative growth of clams in units with and without strips of flexible plastic netting was assessed in the longer-term study. Relative growth =  $([Final\ SL - Initial\ SL] / Initial\ SL) \times 100\%$ . A relative growth value of 100% indicates a doubling in SL.

#### Experiment III b

Another assumption is that the strips of netting used to enclose juvenile clams within experimental units ( $A = 0.0182$   $m^2$ ) do not affect their growth. On May 22, 2003, a generalized completely randomized block design (sensu Underwood 1997) was established at the mid intertidal of the mudflat in Cutler, Maine (see earlier). Eighty experimental units were filled with ambient sediments and pushed into the mud in blocks of four units each. Ten blocks contained units with strips of netting surrounding its periphery and extending above the sediment surface 4–5 cm (as described earlier). The remaining 10 blocks contained units without any strips of netting. Twelve hatchery-reared individuals ( $660$   $m^{-2}$ ) were added to two of the units within each block, whereas the other two received 24 clams ( $1320$   $m^{-2}$ ). Mean initial SL was  $12.9 \pm 0.4$  mm ( $n = 100$ ). On 29 August 2003, the contents of each experimental unit were sieved using a 2-mm mesh and the initial and final SL of each live clam measured to the nearest 0.1 mm using Vernier calipers. ANOVA was conducted on the untransformed mean relative growth using a linear model similar to that described (see **Experiment II: Addison**) earlier except:

$A_i$  = Rim netting ( $i = 2$ , with and without; factor is fixed);

$B_j$  = Intraspecific clam density ( $j = 2$ , 660 versus 1320  $m^{-2}$ ; factor is fixed); and,

$C_k$  = Block ( $k = 10$  blocks per rimming treatment; factor is random).

TABLE 3.

Analysis of variance on the untransformed mean final SL of hatchery-reared individuals of *M. arcuaria* from 10 May to 21 November 2001 at four lower mid-intertidal sites in Addison, Maine. Ten clams ( $12.4 \pm 1.4$  mm SL) were added to protected (netting = flexible, 6.4 mm aperture) and unprotected experimental units ( $A = 0.0182$   $m^2$ ) filled with ambient sediments and arrayed in six blocks per location. ( $n = 2$  or 1 depending on survival.)

Source of Variation	df	SS	MS	F	Pr > F
Site	3	525.13	175.04	12.57	0.0001
Protective netting	1	13.27	13.27	1.41	0.2541
Site $\times$ Netting	3	31.44	10.48	1.11	0.3756
Block (Site)	18	250.63	13.93	1.66	0.1120
Netting $\times$ Block (Site)	15	141.52	9.43	1.12	0.3820
Error	28	235.28	8.40		
Total	68	1197.27			

## RESULTS

## Experiment III a

All 120 clams were recovered alive from the experimental units within the tank at BIRSH, and none had escaped the enclosures.

In the short-term field trial, a small proportion of juvenile clams were missing (mean missing rate =  $4.5 \pm 1.9\%$ ;  $n = 40$ ). Missing rate was unaffected by stocking density ( $F = 1.17$ ;  $df = 1, 32$ ;  $P = 0.4187$ ) or the presence of the strip of flexible plastic netting affixed to the outside circumference of each enclosure ( $F = 0.13$ ;  $df = 1, 32$ ;  $P = 0.5625$ ). Of those experimental units rimmed with netting and further surrounded by a plastic ring, only one live clam of the 180 transplanted to those units ( $24 \times 5$  reps +  $12 \times 5$  reps), or  $<1\%$ , had migrated from the unit (i.e., was found within the sediments outside the unit but within the area delineated by the plastic ring). For units without plastic strips of netting five clams were found in areas delineated by the ring. Two were alive ( $1.1\%$ ) and 3 were dead (2 with undamaged valves and 1 with crushed valves).

In the 100-day field experiment, the rate of missing clams pooled across all treatments was  $24.7 \pm 3.2\%$  ( $n = 40$ ); however, as in the case for the short-term trial, missing rate was unaffected by the presence of the strip of flexible netting ( $\bar{x}_{\text{no netting}} = 27.1 \pm 5.1\%$  versus  $\bar{x}_{\text{netting}} = 22.3 \pm 3.8\%$ ;  $n = 20$ ;  $F = 0.23$ ;  $df = 1, 32$ ;  $P = 0.6338$ ). The addition of a ring of plastic around experimental units helped determine the fate of missing clams. Only one live clam was sampled outside an experimental unit and within the area surrounded by the plastic ring (from an experimental unit without a strip of netting and stocked initially with 24 clams), whereas 40 clams were found dead, with crushed or chipped valves (typical of crustacean predation) in the same area. By adding the ring of plastic surrounding experimental units, the rate of missing clams dropped from  $36.0 \pm 3.4\%$  (units without rings,  $n = 20$ ) to  $13.3 \pm 3.9\%$  (units with rings,  $n = 20$ ) ( $F = 29.09$ ;  $df = 1, 32$ ;  $P < 0.0001$ ). Stocking density did not influence rate of missing clams ( $F = 3.92$ ;  $df = 1, 32$ ;  $P = 0.0565$ ).

Clams grew similarly in units with and without strips of netting ( $P = 0.9948$ ). Mean relative growth in experimental units with strips of netting was  $99.1 \pm 1.9\%$  ( $n = 40$ ) whereas growth in units not surrounded by the rim of netting was  $99.7 \pm 2.7\%$  ( $n = 38$ ). In addition, no density effects on growth were detected ( $P = 0.8174$ ).

## Experiment III b

No significant difference in relative clam growth was found between experimental units with ( $99.1 \pm 3.83\%$ ,  $n = 40$ ) versus without the rim of netting ( $99.7 \pm 5.56\%$ ,  $n = 38$ ) used to enclose clams ( $P = 0.9948$ ; Table 4). Mean final SL of clams in units with netting strips was  $25.3 \pm 0.51$  mm versus  $25.4 \pm 0.57$  mm for clams in units without the strips. In addition, stocking density had no effect on relative clam growth.

## Experiments IV–VI

## Predation Studies

Predation is the single most important and significant factor affecting survival of juvenile clams in eastern Maine (Commuto 1982b, Beal 1994, Beal et al., 2001, Beal and Kraus 2002, Beal in press). Efforts to deter predators using plastic netting have been used recently; however, when the exotic European green crab,

TABLE 4.

Analysis of variance on the untransformed mean relative growth of hatchery-reared juveniles (mean initial SL =  $12.9 \pm 0.4$  mm) of the softshell clam, *Mya arenaria*, from 22 May to August 29, 2003 at an intertidal mudflat in Cutler, Maine. Experiment was designed to assess the interactive effects of stocking density (660 versus 1,320  $\text{m}^{-2}$ ) and the presence or absence of strips of mesh netting (4.2 mm aperture) surrounding the periphery of experimental units on clam growth.

Source of Variation	df	SS	MS	F	Pr > F
Rim of Netting	1	0.000	0.000	0.00	0.9948
Density	1	0.001	0.001	0.05	0.8174
Netting $\times$ Density	1	0.047	0.047	2.47	0.1331
Block (Netting)	18	0.441	0.025	1.17	0.3288
Density $\times$ Block (Netting)	18	0.340	0.019	0.90	0.5775
Error	38	0.794	0.021		
Total	77	1.623			

( $n = 2$  or 1 depending on clam survival).

*Carcinus maenas* (L.), became a nuisance and major threat to clam populations in Maine during the 1950s, chicken wire (spread out over the flats and established as 0.5–0.75 m tall fences with a flange on top) was used to slow down and reduce the effectiveness of this crustacean predator within the intertidal zone (D. Wallace, Brunswick, ME, pers. comm.). Green crabs have been implicated as the cause of the sudden decline of the Maine softshell clam fishery during the 1950s (Glude 1955, Grosholz & Ruiz 1996; Fig. 1). Another major predator of *M. arenaria* in eastern Maine is the moon snail, *Euspira heros*. According to Commuto (1982b), this naticid gastropod preys on *Mya* until clams reach 30 mm SL, then mortality caused by *E. heros* is much reduced.

Here, results are presented from three field studies in eastern Maine to assess the importance of moon snail and green crab predation on wild and cultured individuals of the softshell clam.

## METHODS

## Euspira studies—Experiment IV August 1986 to June 1987

Because Commuto's (1982b) analysis of moon snail predation on softshell clams was indirect (age-specific survivorship estimates and size distributions of living and dead animals from samples taken near the high tide mark of a flat in eastern Maine on three dates), a field experiment was designed to test directly whether clams  $>30$  mm SL attain a size refuge from *E. heros* attack. The intertidal study site, Hinckley Point ( $44^{\circ}54.68'N$ ;  $67^{\circ}12.21'W$ ), is at the confluence of the Dennys and Hardscrabble River near Dennysville, Maine. This site is approximately 12 km (Euclidean distance) from the site in Lubec (Federal Harbor;  $44^{\circ}51.34'N$ ;  $67^{\circ}04.72'W$ ) where Commuto (1982b) conducted his sampling. Wild softshell clams (15–51 mm SL) were dug from the immediate vicinity of Hinckley Point and 94 or 188 uniquely marked individuals were added to 0.25  $\text{m}^2$  plots ( $n = 4$  replicates per density treatment) on August 4, 1986. Plots were marked with a wooden stake in each corner. Clams were grouped into four discrete size classes, which represented the natural distribution of clams at Hinckley Point (15–20 mm = 21%; 21–30 mm = 47%; 31–40 mm = 16%; 41–51 mm = 16%), and pushed into the sediments far enough to cover the posterior margin of each individual. Plots were revisited on June 11, 1987, when all live and

dead clams were removed and measured (SL) to the nearest 0.1 mm with Vernier calipers. Clams that had been attacked by moon snails had an obvious countersunk hole (or sometimes two) usually near the umbo of one of the valves. The mean borehole diameter from each dead clam was measured using a dissecting microscope with an ocular micrometer by measuring the greatest diameter of the hole and then taking a second measurement perpendicular to the first.

Because of the large number of dead clams with drilled valves, a laboratory trial was designed to determine the relationship between moon snail size and the diameter of the borehole it drills in the valves of its softshell clam prey to estimate size of predator in the field experiment. The study was conducted at BIRSH in late summer 1987. Twenty-one *Euspira* (SH = spire to apex; size range = 9–24 mm) were collected from Weir Cove (44°48.70'N; 67°08.26'W), an intertidal flat in Whiting Bay, approximately 12 km from Hinckley Point. One moon snail and five clams (size range = 16–52 mm SL) were added to each of 21 plastic boxes (10 cm × 10 cm × 6 cm deep) containing poorly sorted beach sand to a depth of 5.5 cm. Each box was covered with nylon window screening and individual containers were placed in running seawater (temperature = 14–15°C) for a two-week period. At the end of the trial, all *Euspira*, *Mya*, and boreholes were measured. To increase the scope of this relationship, a second laboratory experiment was conducted beginning on September 10, 1987. Four large moon snails (size range = 57–70 mm) were collected from the lower intertidal at Mill Cove (44°52.58'N; 67°09.71'W), also in Whiting Bay, and 5.2 km from Hinckley Point. One moon snail and ten clams (size range = 27–77 mm SL) were added to each of four 60-L aquaria containing 15 cm of poorly sorted beach sand and placed into running seawater (temperature = 15–17°C). Measurements of predator, prey, and mean borehole diameter were made as described above.

On October 22, 1987, 20 benthic cores ( $A = 0.02 \text{ m}^2$ ) were taken near the upper intertidal at Federal Harbor in the vicinity where Committ (1982b) had sampled from 1977–1979. Core samples were sieved through a 0.5-mm mesh. The SL of each *Mya* with a bored valve and its borehole diameter was measured (as described above), and then the *Euspira*-borehole relationship was used to estimate the size frequency distribution of moon snail predators at Federal Harbor.

#### *Euspira* studies—Experiment V, June to September 1993

To assess how moon snails respond to increasing juvenile clam density along a tidal gradient, a field test at a mudflat near Bell Farm Cove in Edmunds, Maine (Whiting Bay; 44°49.33'N; 67°09.24'W) was initiated on June 22, 1993. Plastic plant pots (as described earlier) were used as experimental units that were arrayed in a single 5 × 6 block at each of 3 tidal heights (high, mid and low). Within each block, hatchery-reared juvenile softshell clams (mean SL =  $8.9 \pm 0.62$  mm; range = 4.2–15.3 mm) were added to the open enclosures at one of two stocking densities (12 or 24 clams per unit, or 660 or 1320  $\text{m}^{-2}$ , respectively). Five replicates of each of the six treatments ( $a = 3$  tidal heights ×  $b = 2$  densities) were removed from the flat on July 22, August 22 and September 20, 1993. The contents of each experimental unit were sieved through a 2-mm mesh. The initial and final SL of all clams with countersunk boreholes, typical of *E. heros* attack, was measured to the nearest 0.1 mm using Vernier calipers. Mean borehole diameter of each drilled valve was measured using a dissecting

microscope with ocular micrometer. An arcsine transformation was applied to the mean percent dead drilled, and then a model 1 3-factor ANOVA was performed to test for treatment effects. In addition, the *Euspira*-borehole relationship (as described earlier) was used to estimate size of moon snail preying on clams and whether predator size varied as a function of tidal height, stocking density and sampling date.

#### Carcinus studies—Experiment VI, October 1993

*C. maenas* has been presumed to be the major crustacean predator in most field experiments conducted in eastern Maine (Beal 1994, Beal et al. 2001, Beal & Kraus 2002). It is not difficult to identify general mortality agent based on shell damage in soft-shell clams. Typically, highest predation rates can be attributed to crab attack that is inferred from crushed and/or chipped valves. Field investigations have shown that crab predation is highly seasonal and varies along a tidal gradient (Beal et al. 2001). Predation rates caused by large crustaceans are relatively low at all tidal heights prior to August. From August until the beginning of October, rates are the highest observed during the year. Mortality of clams (ca. 12.4 mm SL) caused by crustacean predators was <20% for animals held in open enclosures near the high- and mid-water mark at an intertidal flat near Jonesport, Maine from April 6 to December 13, 1996 (Beal et al., 2001). However, mortality rates varied from 40% to 50% for animals in open enclosures near the low tide mark over the same period. Protective netting enhanced clam survival only at the lowest tidal elevation where difference in mean survival between units with and without the predator deterrent mesh was nearly 30% ( $\bar{x}_{\text{protected}} = 91.1 \pm 4.5\%$  versus  $\bar{x}_{\text{unprotected}} = 63.3 \pm 9.7\%$ ,  $n = 120$ ,  $P < 0.0001$ ).

Beal and Kraus (2002) examined the interactive effects of protective netting, initial clam size and stocking density on clam survival in separate studies at two intertidal locations near Jonesboro and Cutler, Maine. From June 1990 to June 1991, crushing predators accounted for 33.6% and 20.3% of losses of clams initially 8.5 and 11.8 mm SL, respectively, in open enclosures. From April to October 1991, losses of clams initially 14.6 mm from open enclosures ranged from 15% to 20%. Until now, however, identification of which crustacean species attacks juvenile clams in the intertidal has been indirect, based solely on shell damage.

To identify which crushing predators are active on clam flats during periods of tidal inundation, a passive trapping study was conducted at an intertidal flat in Cutler, Maine (see earlier) during October 1993. Vinyl-coated, wire lobster traps (61 cm × 55 cm × 38 cm), baited with salted herring (*Clupea harengus* L.), were placed outside and inside each of five sea grass beds (area of each bed ranged from 150–250  $\text{m}^2$ ). Distance between traps from outside to inside a bed was no more than 15 m. The test was initiated at low tide on October 1 and traps were checked irregularly (on 10 dates) at periods of low water until October 21. New bait was added to each trap on each sampling date, except the last. When a crab was found within a trap, it was measured (greatest carapace width to the nearest 0.1 mm) and its sex recorded. Before releasing trapped crabs, the dactyl from each pair of fourth walking legs was excised. This provided a common mark that I used to distinguish animals caught more than once.

## RESULTS

#### *Euspira*—Experiment IV

Of 1128 clams transplanted initially to the eight field plots in August 1986, only 600 were recovered (47% were missing), and of

these, 215 were alive and 385 were dead. Growth rates were extremely slow with animals >40 mm exhibiting no significant shell growth and animals <40 mm exhibiting increases in SL between 3 and 5 mm. Approximately 77% (296) of the total number recovered dead had been drilled by *Euspira* (Table 5). Moon snails did not demonstrate a density-dependent response to their prey based on total number per plot or initial size ( $G = 2.8$ ,  $df = 3$ ,  $P = 0.43$ ) but did show a preference for clams between 31 and 40 mm SL. A comparison of the initial size distribution versus the size distribution of drilled individuals (Fig. 8) reveals that *Euspira* fed selectively on different size clams ( $G = 84.9$ ,  $df = 3$ ,  $P < 0.0001$ ). Examination of the expected frequencies within the  $2 \times 4$  contingency table revealed that the proportion (6%) of drilled clams in the smallest size category (15–20 mm) is three times lower than expected (18%) and the proportion (38%) of drilled clams between 31–40 mm is nearly double the expected frequency (20%).

The relationship between mean borehole diameter and snail size (Fig. 9), allows one to estimate the size of snails drilling clams in the field (Fig. 10). Moon snails at Hinckley Point flat ranged in size from approximately 10–52 mm in shell height. Nearly 70% of the moon snails preying on *Mya* were between 20–30 mm. Mean snail size  $\pm 1$  SE was  $24.4 \pm 0.30$  mm ( $n = 296$ ). Conversely, moon snails at Federal Harbor were smaller ( $\bar{x} = 11.9 \pm 0.29$  mm), and ranged from 8–29 mm ( $n = 94$ ). The two distributions were significantly different ( $P < 0.0001$ ; G-test of independence).

#### *Euspira*—Experiment V

From June to July 1993,  $71.5 \pm 8.13\%$  ( $n = 30$ ) of the *M. arenaria* juveniles were recovered dead with drilled valves, and this rate increased through time ( $P = 0.0066$ ). There was no difference in mean percent with drilled valves from the final two sampling dates ( $81.7 \pm 4.06\%$ ,  $n = 60$ ;  $P = 0.1403$ ). Mean snail size varied significantly with date, tidal height and stocking density (Table 6). Snails were smaller in June ( $16.5 \pm 1.06$  mm,  $n = 29$ ) than they were in July and August ( $17.6 \pm 0.89$  mm,  $n = 60$ ), decreased in size from the high ( $20.6 \pm 0.94$  mm,  $n = 30$ ) to the mid and low tide mark ( $15.5 \pm 0.52$  mm,  $n = 59$ ) and increased in size with increasing intraspecific clam density (mean SH of snails

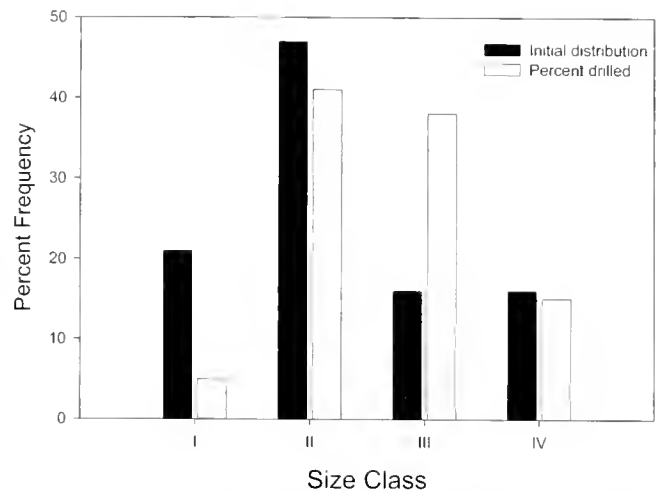


Figure 8. Initial size-frequency distribution of clams used in a field experiment from August 4, 1986 to June 11, 1987 and the distribution of clams with a bored valve from moon snail, *Euspira heros*, attack. Size classes of clams (SL) are: I = 15–20 mm; II = 21–30 mm; III = 31–40 mm; IV = 41–51 mm. The two distributions are different ( $P < 0.0001$ ) indicating that snails prefer a particular size *Mya*. Clams in size class III were drilled at a rate that was nearly double than what was expected ( $P < 0.05$ ; G-test of independence). Conversely, clams in size class I were drilled less frequently than expected.

in units stocked at 660 vs. 1,320  $m^{-2}$  was  $16.5 \pm 0.90$  mm vs.  $17.9 \pm 1.02$  mm).

#### *Carcinus*—Experiment VI

Thirty-one green crabs (mean carapace width, CW = 72.6 mm) and 22 rock crabs, *Cancer irroratus* Say (mean CW = 97.6 mm), were caught in the traps during the test interval. All crabs were male, and no individual was caught more than once. Fewer crabs were caught near the end of the month than at the beginning, and each species demonstrated a preference for one location over another ( $P = 0.02$ ). Green crabs were 4.1× more likely to be caught inside eelgrass beds than outside them, whereas

TABLE 5.

Fate of *Mya arenaria* at two densities ( $1 \times = 94 m^{-2}$ ,  $2 \times = 188 m^{-2}$ ) from 4 August 1986 to June 11, 1987 at Hinckley Point Flat, Dennyville, Maine.  $n$  = number of clams at the beginning of the experiment, A = percent recovered Alive, UV = percent recovered dead with Undamaged Valves, DV = percent recovered dead with countersunk, Drilled Valves, CV = percent recovered dead with Chipped or Crushed Valves. One-way ANOVA on the angular-transformed mean percent with drilled valves (DV) failed to detect significant differences due to stocking density.

PLOT	$n$	A	UV	DV	CV	M
1	94	5	13	39	0	43
2	94	12	6	43	1	38
3	94	12	6	37	0	45
4	94	29	5	9	0	57
1	188	16	6	22	0	56
2	188	30	9	28	1	32
3	188	12	13	29	2	44
4	188	27	2	13	1	57

M = percent Missing.

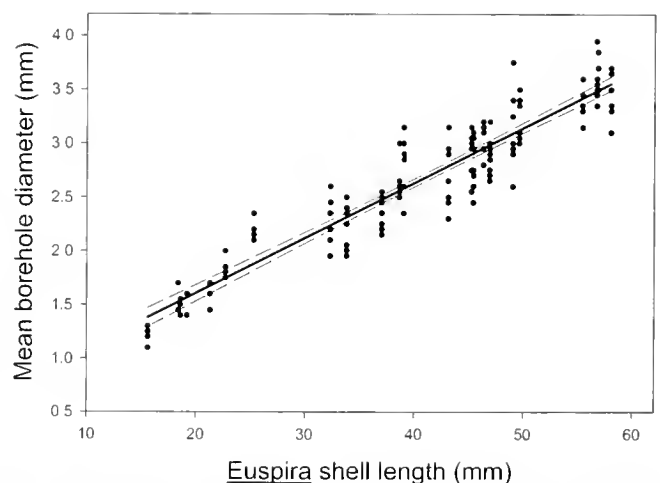


Figure 9. Relationship between mean borehole diameter (in the valves of *M. arenaria*) and size of moon snail predator. Dotted lines represent 95% confidence intervals.  $Y = 0.058 + 0.0512 X$ ,  $n = 138$ ,  $r^2 = 0.884$ ,  $P < 0.0001$ .

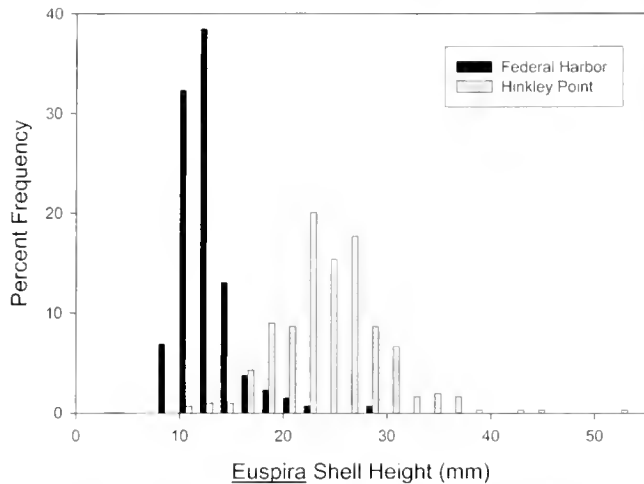


Figure 10. Estimated size-frequency distribution of moon snails from Federal Harbor, Lubec, Maine ( $n = 94$ ), where Commito (1982b) concluded that soft-shell clams reach a refuge from moon snail attack after they attain a SL of 30 mm, and from Hinkley Point, Dennysville, Maine ( $n = 296$ ). Both distributions were created by measuring the mean borehole diameter of clams collected from both sites and then using the linear equation relating borehole diameter to snail size (see legend of Fig. 9).

rock crabs were 2.7 $\times$  more likely to be found in traps on the unvegetated muddy sediments. The results indicate that assigning all crushing and chipping damage to green crabs is not appropriate and that it is possible that rock crabs prey on small softshell clams in the intertidal zone during periods of tidal inundation.

## DISCUSSION

In Maine, USA, intertidal populations of softshell clams are a public resource that is managed by the local community in co-

TABLE 6.

Analysis of variance on the untransformed mean size (shell height) of moon snails (*Euspira heros*) attacking hatchery-reared juveniles of *Mya arenaria* from June 22 to September 20, 1993 at an intertidal flat near Bell Farm Cove, Edmunds, Maine. Clams were stocked at one of two densities (660 or 1,320  $m^{-2}$ ) at three tidal heights (high, mid, low) and samples on three dates: July 22, August 22, and September 23. A decision rule of  $\alpha' = 0.0253$  was used for each single degree-of-freedom, orthogonal contrast involving sampling date and tidal height.

Source of variation	df	SS	MS	F	Pr > F
Sampling date	2	30.03	15.01	3.70	0.0297
(1) June versus July & August	1	29.73	29.73	7.32	0.0086
(2) July versus August	1	0.30	0.30	0.08	0.7846
Tidal height	2	525.69	262.85	64.68	<0.0001
(1) High versus mid & low	1	524.27	524.27	129.01	<0.0001
(2) Mid versus low	1	1.42	1.42	0.35	0.5559
Date $\times$ tidal height	4	27.95	6.98	1.72	0.1552
Density	1	48.56	48.56	11.95	0.0009
Date $\times$ density	2	9.65	4.83	1.19	0.3109
Tidal height $\times$ density	2	5.01	2.51	0.62	0.5425
Date $\times$ tidal height $\times$ density	4	8.28	2.07	0.51	0.7289
Error	71	288.53	4.06		
Total	88	943.70			

( $n = 5$ ).

operation with State government and its regional shellfisheries biologists. Two decades ago, approximately one-half of coastal communities along the Maine coast actively managed their soft-shell clam fishery through local ordinances. Today, that percentage is 70%, and, although most of these communities participate in some form of active management efforts to enhance stocks (e.g., clam transfers, netting to encourage spatfall, planting hatchery-reared seed), all benefit from restricted, or limited entry, which results in an approximate 15% higher yield for harvesters than those in communities without local management plans (Townsend 1985).

## Clam Transfers

Today, approximately 44% of Maine communities that manage their *M. arenaria* stocks participate in some form of clam transfer program (ME DMR 2005) by harvesting clams from areas of high density and replanting them in areas with lower clam abundance (usually lower on the shore). To date, however, only anecdotal information exists about the efficacy of these activities. Generally, seed clams (animals <50.8 mm SL that typically range from 38–45 mm SL) are dug by hand by groups of clambers, and then immediately (or within 24 h) replanted by broadcasting the animals from a boat at high tide into a closed intertidal area. Clams may take up to 72 h to reburrow because burrowing rate depends on clam size and seawater temperature (Zwarts & Wanink 1989, Zaklan & Ydenberg 1997). At present, no shellfish committees apply netting to the reseeded area to deter predators. Further, no community chooses to follow the fate of the transferred animals. Instead, success is measured by the number or volume of clams transferred during any given effort. Although a single field test on clam transfers was conducted and generalizations should not be made, results presented here suggest that if communities choose to participate in clam transfer programs, they can improve production in the reseeded areas simply by using protective netting. This would have to be done at low tide when it would be possible to secure the netting around the seeded plot, however. Netting did not meet the criteria of statistical significance in this study, but did result in an overall 120% increase in final mean density compared with the density of transferred clams in plots without netting. It is unclear, however, whether the netting acted only to retain clams in the seeded areas or improve seed survival by deterring predators. Another benefit of using the plastic, flexible netting was that it enhanced wild recruitment 3-fold compared with control plots.

The transfer field experiment was conducted to provide several biological parameters to help the city of Eastport's shellfish conservation committee decide what steps it should take regarding this management option. No cost-benefit analysis was done; however, in eastern Maine, where large numbers of adult clams occur in the extreme upper intertidal zone, and where growth rates are very slow and legal sizes may be attained only after 10 years (Fig. 4), these efforts may be cost-effective and necessary. Results from this study suggest that growth rate of clams from these high intertidal areas is plastic as they assume rates of shell accretion that is typical of the area to which they are transferred (see also Dow & Wallace 1961). Transfer efforts may allow some communities to harvest clams legally that they may not be able to do otherwise. Typically, clams in these high intertidal areas are not harvested and left to grow and presumably reproduce. It is unknown, however, whether these "refuge populations" act as natural spawner sanctuaries (sensu McCay 1988), and if clam transfer efforts were to increase

whether this would have any effect on natural recruitment in that region.

### Shell growth

Growth of infaunal and epifaunal bivalves in the intertidal zone typically is related to submergence time, varying inversely with tidal height (Mead & Barnes 1904, Newcombe 1935, Jordan & Valiela 1982, Vincent et al. 1989, Stiven & Gardner 1992). Several investigations, however, have shown that although bivalve growth may be faster lower on the shore than in the upper shore area, rates do not necessarily parallel directly submergence times (Peterson & Black 1987, 1988, Beal et al. 2001). The yearlong study at Carrying Place Cove in Eastport (April 1998–1999) demonstrated that clams at the mid and upper intertidal zones grew faster than animals at the extreme low intertidal. Only 5 of 216 clams planted in open plots near the low intertidal survived (2.3%), and these grew extremely slow (e.g.,  $1.9 \pm 1.5$  mm SL). Survival was significantly higher in plots near the mid and upper intertidal, where growth rates followed expected patterns (Fig. 4). Why was growth so slow among clams in the lower intertidal zone where they were submerged nearly 100% of the time? Intraspecific competition can be ruled out because survival rates were so low. Interspecific competition may explain partially this result, because the plots were adjacent to subtidal beds of blue mussels, *Mytilus edulis* L. Another factor may have been disturbance caused by the predatory activities of the moon snail, *Euspira heros*. After measuring the borehole diameters of the drilled clams and using the *Euspira*-borehole diameter equation (Fig. 9), it was determined that the mean snail size preying on the clams at the mean low tide level was 40.0 mm. Reductions in shell growth have been attributed to predators in several field studies. For example, the shell growth of hard clams, *Mercenaria mercenaria* (L.) was nearly 100% slower when animals were exposed to whelk attack (Nakaoka 2000). Similarly, Peterson and Black (1993) observed a 50% reduction in growth of *Katelysia scalarina* Lamarek and *K. rhytiphora* Lamarek in full cages versus open enclosures in Western Australia that was presumably because of increased disturbance by a predatory seastar that had gained entrance to the protected cages.

The growth of juvenile softshell clams in protected and unprotected experimental units was followed in experiments conducted in Jonesport and Addison, Maine. Neither test demonstrated a significant difference in final mean SL between the two treatments, a result consistent with Beal and Kraus (2002). Results from the Addison study indicate the importance of spatial variability in clam growth rates as juveniles at two of the sites exhibited significantly faster growth (20% difference in mean SL) than those at the other two sites. Differences in final SL were not associated with variation in tidal height as experimental units at all four sites were deployed at a single tide level. Spatial differences in bivalve growth may be related to differential microalgal concentrations in the water column that have been found to differ dramatically between adjacent sites (Posey et al. 2002, Carmichael et al. 2004), a reflection of spatial differences in biological disturbance, or differences in sediment composition (Newell & Hidu 1982).

A small percentage of clams in the Addison study attained legal size in one growing season (May to November). From May to November 2001, clams added an average  $27.2 \pm 1.17$  mm ( $n = 22$ ) and  $25.5 \pm 1.71$  mm ( $n = 22$ ) of new shell at Eastern Harbor and Three Brooks, respectively. These growth rates are faster than

reported for this species anywhere in Maine (Dow & Wallace 1953, Spear & Glude 1957, Newell & Hidu 1982, Commito 1982b, Beal 1994). In addition, these rates are faster than those reported for 0-y class individuals of *Mya* in northern Massachusetts (Brousseau 1979).

Previous field studies with hatchery-reared juveniles of *M. arenaria* (Beal 1994, Beal et al. 2001, Beal & Kraus 2002) assumed that the rim of netting used along the periphery of the small experimental units to enclose clams (but still allow predators access to the clams in the unit) did not affect clam growth. Although results presented here and elsewhere (Beal et al. 2001) have demonstrated that completely covering the top of experimental units with protective netting has no significant effect on clam growth, Experiment III a & b tested the assumption explicitly. Those results revealed that published growth rates are not confounded by the addition of a strip of netting projecting 4–5 cm above the surface of the sediments. Relative growth rate and final mean SL were nearly identical in both tests.

### Predation

The three field experiments involving moon snails and green crabs add to an extensive literature on the importance of predation in controlling populations of juvenile and adult softshell clams in Maine (Commito 1982a, b, Thiel 1997, Ambrose et al. 1998, Beal et al. 2001, Beal & Vencile 2001, Beal & Kraus 2002, Whitlow et al. 2003). Although moon snails, *Euspira heros*, occur along the entire coast of Maine, their numbers (as estimated by their predatory activities associated with *Mya*) seem to be greater in eastern Maine than elsewhere (Beal, pers. obs.). Softshell clam seeding activities using cultured juveniles produced at BIRSH that have occurred in each coastal county of Maine (1987–2005) and mortality caused by moon snails is problematic only in eastern Maine (Washington County). By analyzing size-frequency data of both predator and prey populations at Federal Harbor in Lubec, Maine from 1977–1979, Commito (1982b) demonstrated that soft-shell clams escape moon snail predation by growing relatively quickly (over 5 y) to a refuge size of 30-mm SL. Moon snails at the upper intertidal of Federal Harbor rarely exceeded 30 mm in shell height.

On numerous occasions in 1984 and 1985 while inspecting intertidal flats in Cobscook and Whiting Bays, many dead, drilled *Mya* were discovered that were >50 mm SL. The experiment at Hinckley Point from 1986–1987 demonstrated that an absolute size refuge of 30 mm from moon snail attack may be common at some intertidal flats, but not at others (Fig. 10), because moon snail size may vary along a tidal gradient. *Euspira* occurs both in the intertidal and subtidal in Maine waters. Larger moon snails (up to 100 mm in shell height) are found below mean low water, whereas smaller animals are found from mean low water to the high water mark (B. Beal, pers. obs.). It is likely that a gradient of refuge sizes exist for *Mya* from *E. heros* attack that increases in SL from the upper to lower shore levels. Besides the Hinckley Point study that indicated more clams in the 31–40 mm size range fell victim to moon snails than was expected by random chance alone, high mortality rates occurred in clams >30 mm SL in Eastport (Experiment II) at the lowest tidal level from 1998–1999. Subsequent analysis of mean snail size using the *Euspira*-borehole diameter relationship (Fig. 9) showed that snails preying on clams at that site averaged 40 mm in shell height. Similar analysis of boreholes in drilled *Mya* from the Hinckley Point experiment showed that some moon snails were as large as 52 mm in shell height.

Moon snails are voracious predators of juvenile softshell clams. The study conducted at Bell Farm Cove in 1993 demonstrated that approximately 70% of juveniles <10 mm SL were preyed on by *E. heros* during the first month, from June to July. Although mean snail size was <20 mm at all tidal heights at that site, animals were significantly larger nearer the high versus mid and low tide levels. Maximum snail size was estimated to be 30 mm SH. Vencile (1997) determined that moon snails drill clams that are approximately similar in SL to their shell height. In this study, a similar relationship existed (Snail size =  $10.37 + 0.689 \times \text{SL}$ ,  $n = 1262$ ); however, clam SL explained only 6% of the variation in snail size.

Green crabs, *C. maenas*, are perhaps the best known, and most widely studied, predator of juvenile and adult soft-shell clams in Maine and the northeast United States (Glude 1955, Ropes 1968, Hunt & Mullineaux 2002, Whitlow et al. 2003, Hunt 2004). Intertidal field studies in eastern Maine have demonstrated the efficacy of excluding large crustaceans from experimental units containing softshell clam juveniles (Beal 1994, Beal et al. 2001, Beal & Kraus 2002). For example, between April and September, clam survival in the low intertidal zone at Flake Point Bar, Jonesport, Maine varied from 83% to 103% higher in protected versus open experimental units (Beal et al. 2001). Mean losses exceeded 50% in open enclosures, and, because *C. maenas* was found in some open enclosures, this predator was assigned as the mortality agent. The trapping study conducted in Cutler, Maine reveals that *Cancer irroratus* does forage intertidally during periods of tidal inunda-

tion. It remains to be seen whether differences in *Mya* shell damage exist between these two large crustacean predators.

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## TRENDS IN MAINE SOFTSHELL CLAM LANDINGS

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**ABSTRACT** The soft-shell clam, *Mya arenaria* (L. 1758), has the highest landed value of Maine bivalves. Landings have been variable over the last century with current landings one third of their historical highs caused by low harvests in Eastern Maine. Diminished clam populations have been subjected to constant fishing pressure and heavy predation by green crabs apparently unchecked by winterkill. Clam stocks previously closed to harvesting because of pollution are now dug. Reduced breeding populations are tasked to produce a sufficient set to overcome offshore dispersal of larvae to repopulate flats on the coast of Maine. On-bottom and off-bottom sampling found few clam larvae in Eastern Maine. Recovery of the fishery will require reestablishment of breeding stocks.

**KEY WORDS:** clam landings, Gulf of Maine, green crabs, shellfish management.

### INTRODUCTION

The softshell clam *Mya arenaria* supports the major bivalve industry in the State of Maine with a landed value of \$16.61 million dollars in 2004. Since the early 1980s clam landings have declined by two-thirds. Washington County produced about two-thirds of the State landings in the 1970s but decreased to between 20% to 45% of landings in recent years (see Fig. 2 later).

Trends in landings seen in Figure 1 and Figure 2 comparing southern (S) versus eastern (E) Maine, do not show a regular cyclical trend in landings statewide, although Washington and Hancock counties have had two distinct peaks in landings over the past 50 years.

### Management and Regulation

In 1901 Maine authorized towns to license diggers and regulate digging. Most soft-shells were canned. From 1901 to the 1930s, the State limited the canning season from September 15 to June 1. Shipping live clams out of state was prohibited. Summer digging was banned until 1937 when the three counties in the southwest lifted a ban because of the demand for live and shucked clams throughout the region (Fig. 1). In 1947 restrictions on summer digging and transporting clams were lifted in Lincoln County and restrictions in the rest of the State were removed in 1949. By 1958, only 10% of the catch was canned (Wallace 1997).

A minimum harvest size has been used as a clam conservation measure throughout the region. A two-inch minimum size law was passed by Maine in 1935 and repealed in 1963 (Fig. 1) when Maine authorized coastal municipalities to establish local ordinances to regulate digging and implement conservation measures. The number of towns with a local clamming ordinance increased to 45 in 1988 and 74 in 2003. In 1984, the 2-inch minimum harvest size was reestablished by Maine. Massachusetts has had a 2-inch softshell minimum for harvesting since the early 1900s (MacKenzie 1997). In the Bay of Fundy, New Brunswick and Nova Scotia both have a 44 mm (1.75-inch) minimum harvesting size (Jenkins et al. 1997).

Fishing pressure on clams is limited by the number of harvesting licenses issued. The number of licensed diggers increased from 500 in 1888 to 2,600 in the late 1940s after World War II (Fig. 1). The number of licenses remained relatively constant until an in-

crease in the number of licenses in the 1970s corresponded with an increase in catch. Although catch had decreased by 1991 to levels not seen since 1959, the number of licenses decreased but remained above the number of licenses issued before the 1970s (Wallace 1997). Between 1990 and 2001, Cumberland and Lincoln counties issued the most licenses in S Maine with average clam harvest per license of 1,284 and 800.0 lb/license, respectively. In E Maine, despite the decline in landings in the early 1990s, the number of licenses remained relatively constant resulting in a lower catch per unit effort of 469 and 525 lbs per license in the same period. The recent increase in landings in Washington and Hancock counties (Fig. 1 & 2) in the mid 1990s has been matched by an increase in the number of licenses issued in these counties from 1,268 in 1996 to 1,940 in 2001. Consequently, catch per unit effort in these counties has remained low (Fig. 3).

Effort dedicated to clam management varies between municipalities. In 2002 Maine Department of Marine Resources data for appropriations for management and clam resource value for towns, the correlation between expenditures for management and value of clam landings was 0.41 ( $P < 0.01$ ,  $n = 54$ ). Of towns that listed expenditures for clam management, E Maine towns averaged \$443 for management appropriations in 2002, whereas Southern and Midcoast Maine averaged \$1206.

### Pollution Closures

In 1946, Maine closed polluted waters under the National Shellfish Sanitation Program (Fig. 1). In 1974, 3,420 residential direct discharges of sewage had been identified. This number had decreased to 2,446 in 1992, but the number of prohibited acres closed to clamming peaked at about this time. Between 1985 and 1993, the total acreage closed to clamming increased (Fig. 4), whereas landings went down correspondingly perhaps because of a restricted fishing area (Wallace 1997). Before 1990, the DMR estimated that approximately one-third of all productive clam flats in Maine were closed because of pollution (Farrey et al. 1997). Opening flats resulted in an immediate increase in landings. Acreage closed to harvesting, decreased from 270,444 in 1993 to 153,847 acres in 2002; most of the closed acreage was in the S Maine. In New Brunswick, Canada about half of the flats were closed because of pollution in the mid 1990s and these closures were considered relatively permanent (Jenkins et al. 1997).

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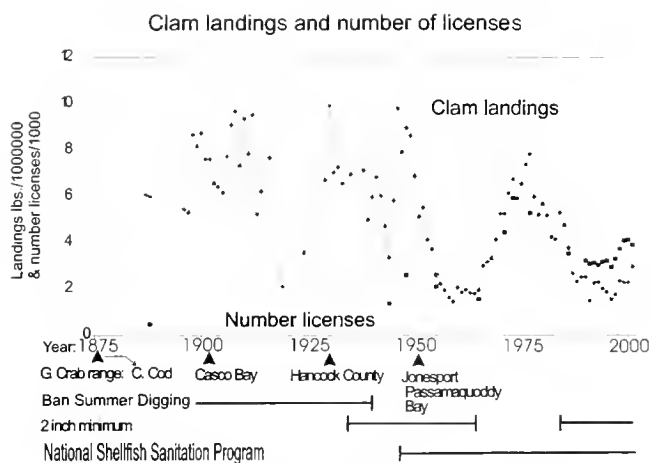


Figure 1. Maine softshell clam landings by year with northern limit of green crab range and years of implementation of management practices. Rectangles plot number of clam licenses issued.

#### Gulf of Maine Tidal Dispersion

Tides and tidal currents in Gulf of Maine increase to the north and east. The tidal range is 3.4 m in Barnstable Harbor, on north side of Cape Cod, increasing to 6.5 m in Eastport in E Maine. Tidal flushing can be measured by determination of an exchange ratio and/or flushing time. The tidal exchange ratio is the ratio of bay volume at high tide to the total tidal flow. Flushing time is time required to replace the total volume of water. Flushing time decreases and exchange ratio increases with tidal amplitude.

Barnstable Harbor is at the southern end of the Gulf of Maine. In the 1940s and 1950s, the clam populations in the harbor decreased to negligible proportions and efforts to reestablish were largely unproductive (Ketchum 1954). Using a conservative exchange ratio for Barnstable Harbor of 0.3 (tidal volume 30% of bay volume), Ketchum (1954) estimated ~0.01% of the larvae spawned in the harbor would be retained to set. Offshore flushing was identified as the likely reason that "only rarely indeed is a substantial set obtained."

Tidal flushing would move clam larvae away from the mudflats where they are spawned. Edwards & Sharples (1986) estimated flushing times for 110 Scottish lochs using tidal range, low water residual volume and the water surface area at low and high water.

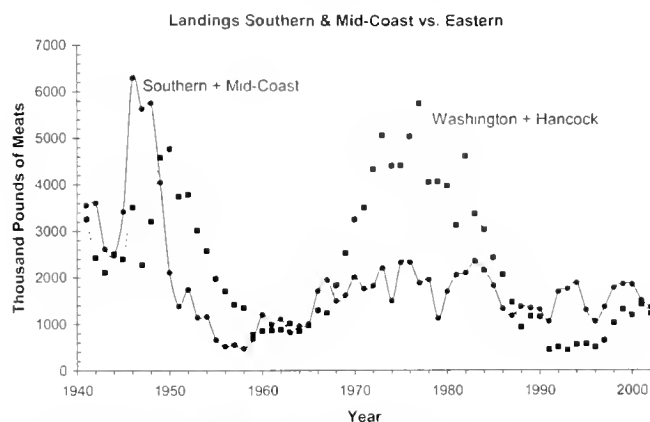


Figure 2. Eastern (Washington and Hancock) and southern and mid-coast Maine softshell clam landings by year.

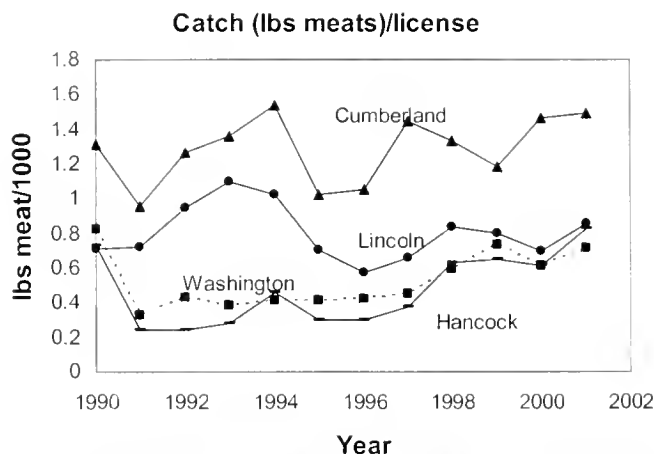


Figure 3. Catch per license issued in counties in Southern Maine (Cumberland and Lincoln) and Eastern Maine (Washington and Hancock).

Flushing times varied from <0.5 to 14 days, with the shorter flushing times for small, shallow lochs and longer flushing times for deep-sea lochs. Using the same technique, flushing time of Quahog Bay on the mid Maine-coast (mean tidal range of 4.0 m and high water area of 4.3 km<sup>2</sup>) was estimated to be 1.25–1.5 days (Holte et al. 2003).

To determine the effect of a larger tidal range on flushing time, flushing times were determined for two bays in E Maine. Both bays had a tidal range of 5.2–5.3 m, but differed in size: high water area of Mason Bay is 3.7 km<sup>2</sup>; high water area of Englishman Bay is 131.7 km<sup>2</sup>. Using the technique of Edwards and Sharples (1986) flushing times was determined as: 0.281 days for Mason bay and 1.8 days for Englishman bay.

A flushing time of <0.5 is less than one tidal cycle. Mason Bay, with an estimated flushing time of 0.281 days, drains nearly dry on spring tides. Low flushing times and high exchange ratios in Gulf of Maine bays, could be responsible for dispersing larvae offshore away from their native mudflats. Offshore dispersion of clam larvae would be expected to be greater where the tides are larger (i.e., E Maine relative to S Maine). Tidal processes would also be expected to have more effect on larval dispersal in the Gulf of Maine relative to bays and estuaries south of Cape Cod where tides are seldom greater than 1.5 m.

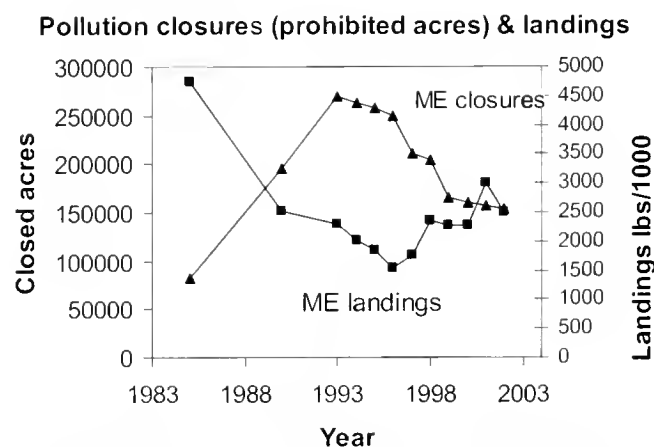


Figure 4. Maine pollution closure (acres) and landings (lbs/1000).

#### Data—Clam Sets Along the Maine Coast

Mudflats in different parts of Maine were surveyed for year 0 juveniles to compare clam sets in different parts of Maine. Core samples from mudflats were collected from E and S Maine in late fall and winter for 2 years and sieved. Juvenile clams (1.8–6.0 mm) from the set of the preceding summer were counted. Densities of juveniles averaged 16.9/m<sup>2</sup> from 120 samples from E Maine and 204.5/m<sup>2</sup> from 120 samples in S Maine (Vassiliev et al. 2000). *Mya* off-bottom settlement was also sampled with spat bags filled with monofilament line in locations in E and S Maine and retrieved monthly. Spat bags placed inside and outside the Scarborough River in S Maine had  $484.4 \pm 972.8$  and  $132.6 \pm 189.5$  juveniles/bag, respectively, compared with  $2.2 \pm 6.4$  and  $1.6 \pm 6.3$  juveniles/bag in the interior and outside of Mason Bay in E Maine. The differences between E and S Maine ( $P < 0.001$ ) were statistically significant by *t*-test. The results of both of these samples suggest that the recent declines in clam harvests in E Maine may be at least partially caused by poor clam sets.

#### Predation—Green Crabs

Green crabs are a major predator of shellfish from newly settled juveniles to adults. Predation by juvenile green crabs and fish was found to be the ultimate factor controlling abundance of *Mya* juveniles in Barnstable Harbor, Massachusetts (Hunt & Mullineaux 2002). Green crabs were scored as very serious shellfish predators (8.3 on a scale of 1–10 with 10 being the most damaging) in southern New England (Walton 2001).

Green crabs were not found north of Cape Cod (Fig. 1, Fig. 5) in 1872 (Smith 1879). By 1905, green crabs were found in Casco Bay in Eagle Harbor, Harpswell and the New Meadows River (Rathbun 1905). By 1930, samples had been collected as far east as Brooklin in Hancock County and by the 1930s, in Frenchmen's Bay and Winter Harbor in Hancock County, but they were not found in Washington County. By spring, 1951, their range extended eastward to Jonesport and Lubec. By the end of the summer

of 1951, they were found throughout Passamaquoddy Bay (Scattergood 1952).

Traps especially designed to catch green crabs were used by the Maine Bureau of Commercial Fisheries to sample crab populations from 1953–1960, and 1965–1967 (Welch 1968). In S Maine, crabs were trapped in Love's Cove, Southport. In 1953, daily trap catches at Love's Cove ranged from 110–670 crabs. Tagging and recapture studies estimated a population of 10,000 crabs per acre (Spear 1955b). Trap catches of green crabs declined in all areas sampled (Table 1) between the mid 1950s and mid 1960's (Welch 1968).

Figure 6 shows surface water temperatures recorded at Boothbay Harbor, Maine over the past 100 years. The years in which large numbers of green crabs were trapped on the Maine coast (Tab.1) were the years with extremely warm winter water temperatures (Fig. 6). Figure 1 and 2 show a decline in clam landings in the early 1950s when crab trap catches were high. In 1955, clam abundance in study areas was reported to have decreased by 50% over 4 years principally in places where green crabs were abundant (Glude 1955). As winter water temperature cooled in the 1960s, numbers of green crabs in E Maine declined sharply (Welch 1968). The fishery recovered in the mid 1960s (Fig. 1 & 2). In S Maine, green crab populations did not reach the low points observed in Eastern Maine and Canada (Welch 1968).

In recent years, sampling of green crabs in Maine has been limited. Green crab sampling was discontinued by the Maine Department of Marine resources (DMR) until 2001. In 1997 and 1998, green crabs were found in two locations sampled on Mt. Desert Island and two of three locations sampled in Casco Bay, but were not found in an estuary in Camden on Penobscot Bay (Whitlatch & Osman 1999). In the fall of 2002, 107 crabs per man-hour search were collected by DMR personnel at 12 sites along the Maine coast (Mercer 2003). This catch/man hour sampled is lower than the highest capture rate reported for Perry, Maine in 1953–1957 of 552/man hour, but higher than the low capture rate of 7/man hour in 1960–1965.

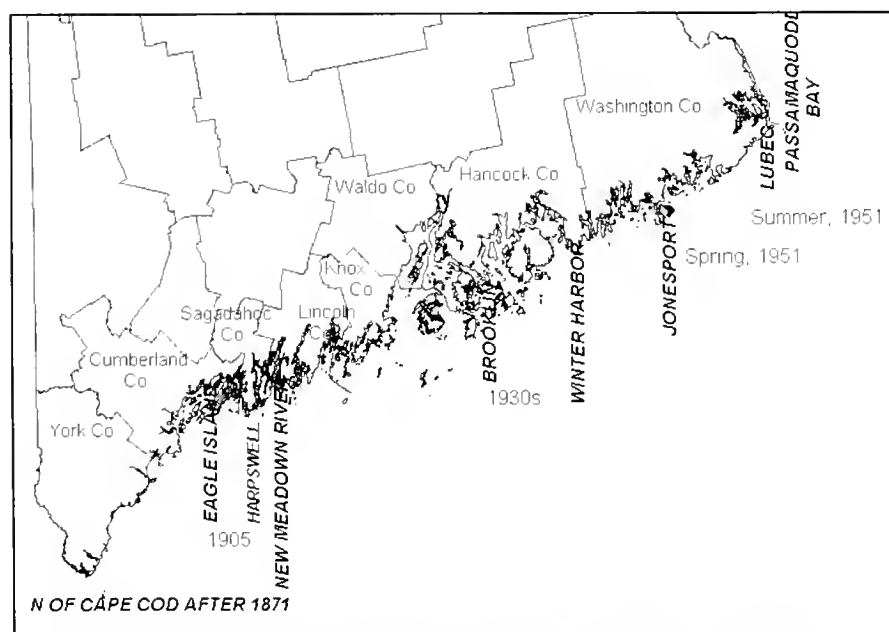


Figure 5. Distribution of the green crab along the Maine coast moving eastward.

TABLE 1.

Green crab sampling between 1953 and 1965 with a standard green crab trap (Welch 1968) except for Perry, Maine where samples are crabs found per man-hour search.

Location	Year of sampling		
	1953–7	1958–9	1960–5
Bocabec River New Brunswick	343	53–41	7.5/trap day
Perry	552		7/man-hour
Lubec	13	16	0/trap day
Jonesport, Cummins Beach	235–500	50	50–6/trap day
Mount Desert I.	255–8	0	0/trap day
Southport, ME	110–670/trap day		

#### Data—Green Crab Observations 2003–2004

In fall 2003 and winter 2004, two locations in E Maine were sampled with commercial crab traps from October 2003 to January 2004 (Jacques et al. 2004). Trap catch/tide ranged from 11–0/trap/tide at Tidal Falls from October to January and 76–0 trap/tide at Bunkers Harbor during the same period. Although the trap design was different from traps used by DMR in the 1950s, the crab catch at Bunkers Harbor in the fall adjusted to 1.9 tides/day was comparable to catch rates throughout Maine in the 1950s in Table 1. During the same fall–winter, four fishermen bottom dragging in E Maine, reported moderate to numerous live crabs in their traps in the fall, but four of five dragging in the spring found numerous dead green crabs in their hauls after a particularly severe winter with an average water temperature of 2.81°C, which is one of the lowest winter water temperatures in 60 years (See Fig. 8 later).

In laboratory, using crabs caught in the field trial, green crabs were immobilized at 1°C, mortality observed at –3°C with larger crabs dying sooner (–0.036 days survival/mm carapace width,  $P < 0.001$ ) at this temperature (Jacques et al. 2004). The temperature threshold for immobility was lower than the 8°C observed by Atkinson & Parsons (1973), but the temperature threshold for mortality was similar to –2.3°C (Spear 1955a). Laboratory observations of immobility at 1°C corresponded with field observations because no crabs were caught when the water was below this temperature. On the Thames River, temperature was found to be

the only physiochemical variable with a significant effect on green crab abundance (Attrill et al. 1999).

#### Maine Coastal Water Temperature and Green Crab Abundance

Because S Maine and E Maine clam populations have shown different trends through time (Fig. 2), separate time series of local water temperatures were obtained for each region. Water temperatures for the Eastern Maine Coast/Bay of Fundy were from the Canadian Atlantic Zonal Monitoring Program (AZMP, 2004), which collects data through a network of sampling locations to provide basic information on the physical chemical and biological properties of the Northwest Atlantic continental shelf. The Prince 5 Station is at 44.9°N latitude and 66.8°E longitude, east of Eastport, Maine, records continuous daily average water temperatures and salinity at a depth of 50 m. Average daily temperatures and salinities for monthly time series were obtained for 1924 through the present from the Bedford Marine Institute.

Records of surface water temperature (1905 to the present) in S Maine were obtained from the Maine DMR Laboratory, Boothbay Harbor (BBHR). From 1905 through 1949, three thermometer readings taken from the station wharf were averaged. From 1950, measurements were taken 1.68 m below mean low water with infrequent measurements taken from the inlets of the flowing water tanks. Salinity was not measured. The correlation between the mean annual water temperature at Prince 5 and BBHR between 1941 and 2001 was 0.437 ( $P < 0.01$ ). This correlation between annual water temperature at these two stations is similar to the correlations both within and between years between different regions of the Gulf of Maine surface waters with 15 significant correlations between 0.47 and 0.62 and 30 correlations  $< 0.46$  (Mountain & Manning 1994).

The average of water temperatures for the preceding 4 years was examined to predict clam landings because a temperature effect on either clam growth or clam predators would affect landings for four subsequent years as individuals that are presently juveniles grow to market size. Green crabs preferentially prey on small clams and the effects of green crab predation may not be seen until the older age classes are not replaced (Floyd & Williams 2004). Annual clam landings in S Maine (York, Cumberland, Sagadahoc, Lincoln, Knox and Waldo counties) and average winter water temperature for the preceding 4 years for the months of January, February and March measured at BBHR are in Figure 7. The minimum water temperature generally occurs between calendar days 70 and 80 (Mountain & Manning 1994), which would be when most of the winterkill of green crabs would occur. Figure 8 contains annual clam landings in E Maine (Hancock and Washington Counties) and preceding 4-year winter water temperature measured at Prince 5.

Temperature time series for southern and eastern Maine display a warm period in the early 1950s as previously noted, a cold period through the early to mid 1960s followed by a warming period in the late 1970s (Fig. 7, 8). These variations in water temperature at two locations agree with the decadal scale temperature variations observed for the shelf-wide volume-averaged eastern Scotia Shelf and central Gulf of Maine data for the upper 300 m (Loder et al. 2001). The relatively abrupt winter temperature decreases observed in late 1970s at BBHR and in the late 1980s at Prince 5 were not seen in averaged data for the entire region.

The relationship between winter water temperature and clam abundance differ in E Maine and S Maine (Fig. 7, 8). The corre-

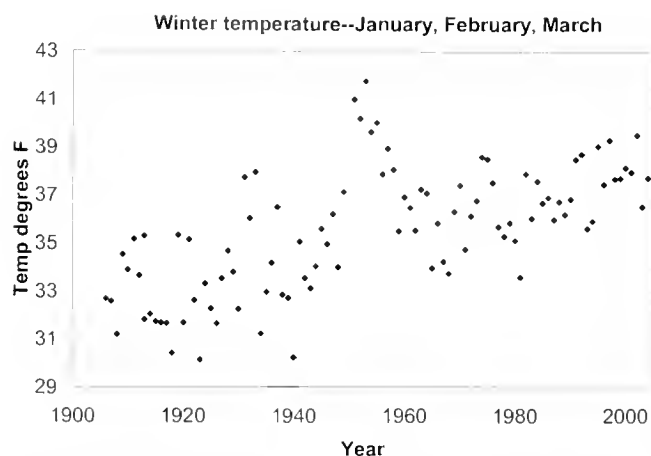


Figure 6. Winter water temperatures (January, February, March) at Boothbay Harbor, Maine.

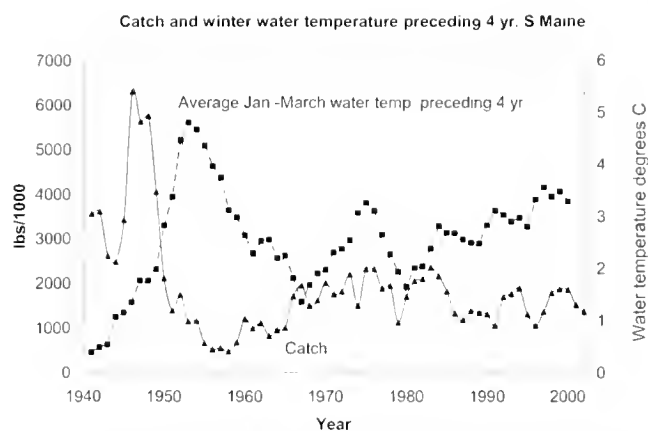


Figure 7. Clam landings (solid line) in southern and midcoast Maine and average water temperature (dashed line) in January, February and March for the preceding 4 years at Boothbay Harbor.

lation between preceding 4-year water and clam abundance is  $-0.53$  in S Maine and  $+0.54$  in E Maine. The colder winter waters in S Maine could cause sufficient green crab winterkill to decrease green crab population size, decrease clam predation and increase subsequent clam landings. As a result, the correlation between winter water temperature (lag up to 4) and clam landings is negative in S Maine ( $r = -0.53$ ).

Eastern Gulf waters do not get as cold as S Gulf waters in the winter because the southwesterly Gulf is less stratified than the eastern Gulf in the winter (Mountain & Manning 1994). Surface waters in winter in E Maine may not usually be cold enough to cause wide spread crab winterkill. The observed effect of warmer winter temperature in E Maine has been to increase landings ( $r = +0.54$ ), possibly caused by a longer growing season for clams and early spring algal bloom. However, extremely cold winters such as that observed in the late 1950s and early 1960s (Fig. 8) could cause high green crab mortality even in eastern Maine. Decreased green crab densities were sampled in eastern Maine during this period (Table 1).

Winter water temperature has been found to impact green crab populations and bivalve predation in other areas. A relationship

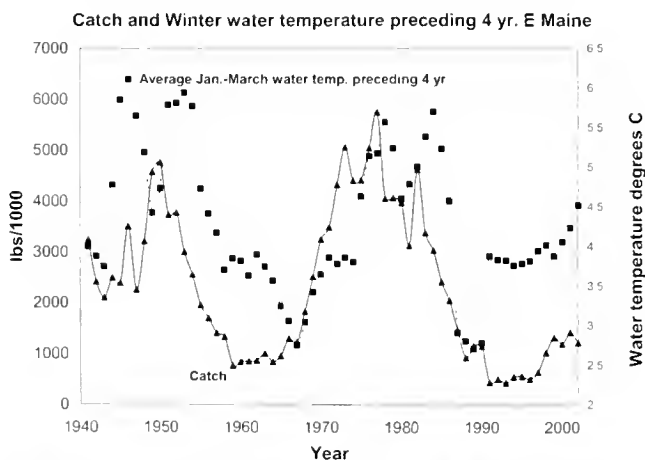


Figure 8. Clam landing (solid line) in Eastern Maine (Hancock and Washington Counties) and average water temperature (dashed line) in January, February and March for the preceding 4 years measured at Prince 5.

between winter temperatures and clam sets has been observed in Europe in areas with green crabs. High recruitment of bivalves after severe winters in temperate shallow waters occurs in some areas of the Wadden Sea (Strasser et al. 2003). Although there are numerous possible explanations for increased larval recruitment after severe winters, reduced numbers of larvae of both *Mya* and *Carcinus* were observed after the winter of 1995/6 "supporting the hypothesis that reduced epibenthic predation is an important factor in high bivalve recruitment after severe winters" rather than enhanced *Mya* larval supply (Strasser & Guenther 2001).

## CONCLUSION

Softshell landings have dramatically declined in Maine over the past 25 y because of low productivity of clam flats in Eastern Maine. Low productivity of clam flats would be affected by:

- high fishing pressure on diminished stocks;
- heavy predation by green crab populations not limited by winterkill in the E Gulf.

Density of clam sets would be decreased by:

- harvesting stocks previously protected by pollution closures.
- Dispersion of clam larvae away from their flats of origin by strong tidal currents.

Sporadic heavy sets of juvenile clams do occur. Epifanio and Garvine (2001) describe north winds, Ekman flow and the resulting shoreward downwelling moving the blue crab, *Callinectes sapidus* (Rathbun) megalopae larvae inshore toward settlement sites in bays along midAtlantic states. Clam pediveligers that move to the surface waters could likewise be moved inshore. The prevailing wind direction in August through November in Portland, ME is from the NNW to NW (National Climatic Data Center, 1998; Fefer & Schettig 1980) and would produce favorable shoreward Ekman flows. The unpredictable occurrence of favorable winds on surface patches of pediveliger larvae would result in settlement events.

The extension of the range of the green crab up the coast of New England has negatively impacted clam populations particularly in areas such as E Maine where warmer winter water temperatures may have limited winterkill of green crabs. Change in the Gulf of Maine Sea Surface temperature (SST) over 120 years was estimated from a NOAA Extended Reconstructed SST data provided by the NOAA-CIRES Climate Diagnostics Center (Clean Air-Cool Planet 2005). The change in mean annual SST between 1880 and 2001 was estimated to be  $0.6^{\circ}\text{C}$  and the change in mean winter temperature over the same period was  $0.2^{\circ}\text{C}$ . Green crab sampling should be used to determine effect of crab populations on clam stocks. If crab populations are determined to be one of the factors limiting reestablishment of clam stocks, techniques for crab control should be developed and implemented (Walton 2000).

Management practices over the past century have not maintained a consistent clam fishery (Fig. 1) and present management practices such as a minimum harvest size have not been successful at reestablishing clam populations in E Maine (Fig. 2).

Management strategies should be implemented to increase harvests and sets:

- Clam mariculture could be used to produce both marketable clams and sets to repopulate local flats. Coastal areas could be managed to produce harvests from both cultivated and wild clams while producing sufficient larvae for a natural set.
- Rotational digging of flats should be evaluated to maintain

breeding populations while allowing adults to grow without breakage and reburial from premature digging. Areas that are dug in the rotation could be repopulated with a natural set, hatchery produced seed or seed from areas with high density sets.

Regional coordination of restoration effort may be required because local spawn may not become the local set.

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## INITIAL RECRUITMENT AND GROWTH OF SURFCLAMS (*SPISULA SOLIDISSIMA* DILLWYN) ON THE INNER CONTINENTAL SHELF OF NEW JERSEY

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**ABSTRACT:** Surfclam (*Spisula solidissima* Dillwyn) larval settlement and the initial growth of recruits were studied on the inner shelf of New Jersey. Initial recruitment was measured by taking weekly benthic core samples during the summer settling season, and larval supply was characterized using microp plankton samples taken every four hours in July. The temporal variation in recruitment at two inshore stations (12-m depth) was linked to larval supply from the water column, and spatial differences (inshore vs. offshore) in recruitment also appeared to be related to larval supply. Spatial and temporal variation in larval concentrations was associated with wind-driven cross-shelf circulation. Contrasting recruitment patterns between the two inshore stations could not be explained by larval supply alone and were likely affected by near-bottom flows. Growth rates of initial surfclam recruits (with initial shell lengths  $<360\text{ }\mu\text{m}$ ) were estimated to be  $10\text{--}20\text{ }\mu\text{m d}^{-1}$ , and the growth rates of individuals  $>360\text{ }\mu\text{m}$  shell length were  $25\text{--}50\text{ }\mu\text{m d}^{-1}$ . This study provides realistic field estimates of early growth rates of surfclams and further evidence of the relationship between upwelling/downwelling events and surfclam larval supply and initial recruitment on the inner continental shelf.

**KEY WORDS:** surfclam larvae, settlement, post-larval growth, recruitment

### INTRODUCTION

The surfclam (*Spisula solidissima* Dillwyn) lives in a zone from the shallow subtidal out to depths of about 60 m, and ranges from the Gulf of St. Lawrence, Canada, south to North Carolina (Merrill & Ropes 1969). It is an important commercial species in the Mid-Atlantic Bight (NOAA/NMFS 2003). Surfclam populations are characterized by great year-to-year variation in recruitment success, especially in inshore areas (Murawski & Serchuk 1989, Weinberg 1993, 1999, Chintala & Grassle 2001). Pre- and postsettlement processes, both physical and biological, are likely responsible for spatial and temporal variation in recruitment success.

To understand factors affecting inshore surfclam larval settlement and initial recruitment a study was initiated at a Long-term Ecosystem Observatory at 15-m depth (LEO-15) off southern New Jersey in 1993. The study area lies within one of the recurrent upwelling centers on the New Jersey coast (Glenn et al. 2004) and is characterized by episodic summer upwelling/downwelling events, which appear to be related to the arrival of high concentrations of surfclam larvae. Studies on larval surfclam supply with respect to these events (Ma & Grassle 2004, Ma 2005, Ma et al. 2006), larval settlement (Snelgrove et al. 1999, 2001, Weissberger & Grassle 2003, Ma 2005) and recruitment during the first year of life (Weissberger & Grassle 2003) form the background for the present study. All of these observations and experiments were focused on larvae spawned by inshore surfclam populations that experience rapidly warming temperatures in the late spring (Starypan 1976). At LEO-15, benthic studies (1993–2004) show that larval settlement from this inshore spawning population occurs chiefly in late June and July, with some markedly reduced settlement in August in some years (Weissberger & Grassle 2003, Snelgrove et al., 1999, 2001, Grassle unpubl. data). Offshore surfclams living under the influence of the Middle Atlantic cold pool do not spawn until the thermocline breaks down in the late summer or early fall (or in some instances when there is storm mixing). It is

likely that these larvae are widely dispersed on the continental shelf in the fall and winter months, and we have evidence of surfclam larvae being present in the plankton at LEO-15 as late as February of the following year (Gregg, Tucker & Grassle, unpubl. data).

This study focused on benthic sampling at three LEO-15 stations during the expected peak in surfclam settlement in July 1998, together with frequent near-bottom sampling of surfclam larvae at the same stations. The year 1998 was chosen in part because initial surfclam recruitment was relatively high for the first time since 1993 (Weissberger & Grassle 2003). The primary goal was to identify the arrival of high concentrations of competent surfclam larvae, and to follow the growth of the consequent pulses of juvenile surfclams in the benthic samples at frequent enough intervals to provide estimates of growth rate in the earliest postsettlement stages (i.e., from a maximum shell length at settlement of approximately  $290\text{ }\mu\text{m}$ ). One goal of our study was to describe surfclam growth during the earliest postsettlement stages, because it is likely that these growth rates will affect whether the juveniles find a size refuge from predation.

### MATERIALS AND METHODS

#### Study Site

The study site was located in the vicinity of Beach Haven Ridge (Fig. 1). Samples were taken from three stations, two inshore (Sta. 9 and C at 12 m depth) and one offshore (Sta. C2 at 20 m depth). Sta. 9 ( $39^{\circ}27.69'\text{N}$ ,  $74^{\circ}15.81'\text{W}$ ) is on the inshore flank of the ridge with a coarse sand bottom, and Sta. C ( $39^{\circ}27.85'\text{N}$ ,  $74^{\circ}15.11'\text{W}$ ) is on the offshore flank of the ridge with a finer sand bottom (Craghan 1995). Sta. C is about 1 km seaward of Sta. 9. Sta. C2 ( $39^{\circ}23.24'\text{N}$ ,  $74^{\circ}12.75'\text{W}$ ) is a sandy site, 8 km offshore from Sta. 9. At Sta. 9 and C bottom temperatures during the summer vary greatly during alternating periods of upwelling and downwelling, whereas the bottom temperature at Sta. C2 rarely exceeds  $12^{\circ}\text{C}$  during the summer months, except for occasional storm mixing (Glenn et al. 2004, Ma & Grassle 2004).

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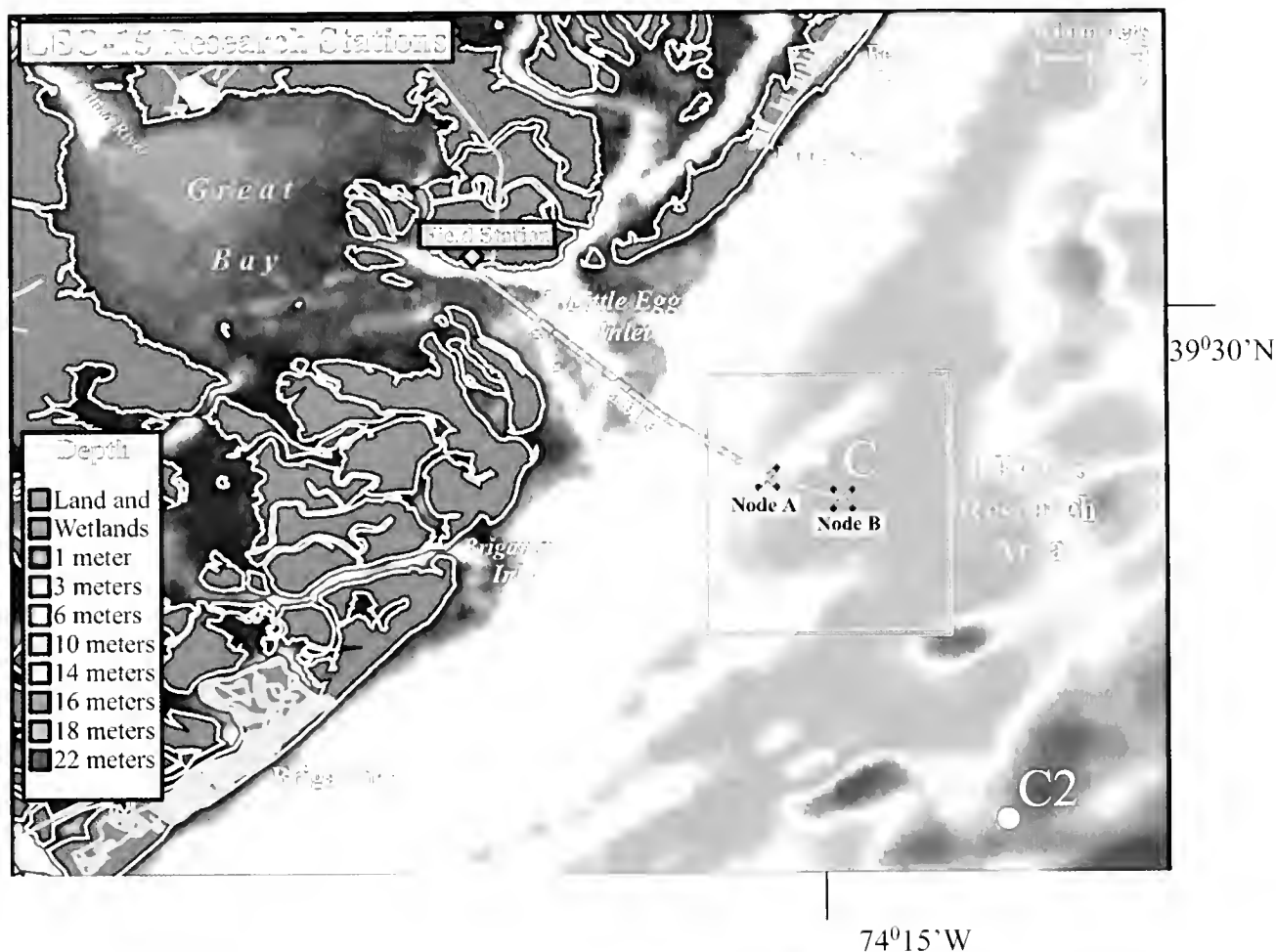


Figure 1. Bathymetric map of LEO-15 and locations of sampling stations (modified from Ma and Grassle 2004).

#### Physical Data

Alongshore wind (SW/NE) and water temperature 1 m above the bottom were used to track upwelling and downwelling events. Wind data were from the Rutgers University Marine Field Station meteorological tower, where wind intensities were recorded 10 m above the ground. Water temperatures were recorded by thermistors on the zooplankton pump housing and on the pump frame.

#### Surfclam Larval Sampling

Three Moored, Automated, Serial, Zooplankton Pumps (MASZPs, Doherty & Butman 1990) were deployed at Sta. 9 and C from July 6–27 and at Sta. C2 from July 11–14, 1998. Samples (250 l) were taken automatically every 4 h, 1 m above the bottom (see Ma & Grassle 2004 for further details). The samples were filtered through 100- $\mu$ m mesh and organisms trapped on the filter were wound onto a take-up spool in a fixative compartment with 10% buffered formaldehyde. The take-up spool was retrieved after a 3-wk deployment. The meshes were cut into individual pieces corresponding to each sample and transferred to 70% ethanol with Rose Bengal. Each sample (no splitting) was examined under a dissecting microscope. Bivalve larvae with shell length  $>150$   $\mu$ m were identified (Ma & Grassle 2004). In samples with high concentrations of surfclam larvae, shell lengths for up to 100 individuals were measured using an ocular micrometer to judge the readiness of the larvae to settle.

#### Benthic Sampling

Divers took 8 benthic core samples (7-cm diameter corer, area 38.5 cm<sup>2</sup>) at Sta. 9, C, and C2 at approximately weekly intervals in July, then at increasing intervals to monthly from September to December 1998. At Sta. 9 and C2 (rippled bottom) four pairs of samples (ripple trough and adjacent crest) were taken, with about 1 m between pairs. At Sta. C (no ripples) paired samples were taken at approximately the same intervals. Core samples were kept on ice for several hours until they were preserved in 95% ethanol and subsequently stained with Rose Bengal. The samples were sieved over a 106- $\mu$ m sieve and sorted under a dissecting microscope. When there were high concentrations of surfclams at Sta. C, a total of 200 individuals (100 haphazardly taken from each of two replicate cores) were measured. At Sta. 9, individuals from 6–8 replicate cores were combined to obtain 200 individuals for each sampling date in July. Initial surfclam recruitment at C2 was too low to achieve comparable sample sizes for measurement. Surfclam images were captured using a color camera linking a Zeiss dissecting microscope to a Macintosh computer and the IMAGE program (calibrated with a micrometer) was used to measure shell lengths.

Surfclam larvae settle at shell lengths from 230–290  $\mu$ m (Snelgrove et al., 1998), and surfclam juveniles were observed to increase in shell length  $\sim 10$   $\mu$ m per day in the laboratory (J. Bell, unpublished data). In the present study surfclams with



shell lengths  $<360\text{ }\mu\text{m}$  were considered to have settled recently, probably within the previous week (a time interval equivalent to the benthic sampling intervals in July). This cutoff assumed an upper limit for settling larvae of  $290\text{ }\mu\text{m}$  and 7 days of growth at  $10\text{ }\mu\text{m d}^{-1}$ . The accumulation of surfclam individuals  $<360\text{ }\mu\text{m}$  was used to estimate initial recruitment with the recognition that this estimation would incorporate early postsettlement mortality.

#### Data Analysis

A Computer-Assisted Analysis of Mixtures (C.A.MAN, Böhning et al. 1992) was used to find the number of cohorts and the mean shell lengths for each cohort. On each date/station combination, shell distributions were fitted to different numbers of cohorts. In general, the greater the number of cohorts included for fitting, the larger the likelihood of the fitting. Decisions on choosing the number of cohorts were based on likelihood adjusted by the number of estimated parameters (i.e., Akaike's Information Criterion, Weissberger & Grassle 2003) and visual fitting with the raw data in histograms. The initial values (mean and weight of each cohort) were estimated from raw data, and C.A.MAN calculated the maximum likelihood estimates for these parameters. Growth was estimated by following the cohorts over time. Urban (2002) pointed out that bivalve larvae and postlarvae grow linearly or exponentially whereas adult growth is asymptotic. Growth rates for the surfclams were calculated assuming both linear ( $L_{t+\Delta t} = L_t + r \times \Delta t$ ) and exponential growth ( $L_{t+\Delta t} = L_t \times e^{r\Delta t}$ ).

Analysis of variance was carried out for densities of initial

surfclam recruits ( $<360\text{-}\mu\text{m}$  shell length) in July using a general linear model (GLM) in SAS. Dates and stations were fixed factors. The GLM procedure was used instead of ANOVA because of the variable number of replicate samples (6–8) completed for each station/date combination (Cody & Smith 1997). The data for number of surfclams were logarithmically transformed to remove heterogeneity in variance. Because of the unbalanced design, standard multiple comparison tests (such as Tukey, Duncan, or SNK tests) were not used (Cody & Smith 1997). Instead, multiple *t*-tests with an overall significance level  $\alpha (=k \times \alpha') = 0.05$  were used, where *k* is the number of comparisons and  $\alpha'$  is the significant level for individual *t*-tests (Bonferroni method).

## RESULTS

### Physical Conditions and Surfclam Larval Concentrations in the Water Column, July 1998

Downwelling winds were interspersed with episodic upwelling winds before July 12, and bottom temperatures at the two inshore stations, Sta. 9 and C, varied between  $13^\circ\text{C}$  and  $17^\circ\text{C}$  until July 10 when temperatures increased to around  $20^\circ\text{C}$  from July 11–13 (Fig. 2). Upwelling winds began July 12 and persisted until late on July 24 when downwelling winds blew for 2 d. During the sustained upwelling wind, bottom temperatures were  $<13^\circ\text{C}$  for  $>10$  days. Temperatures increased near the end of the larval sampling period during the 2 d of downwelling winds. At offshore Sta. C2, bottom temperatures remained  $<12^\circ\text{C}$  throughout July (Fig. 2).

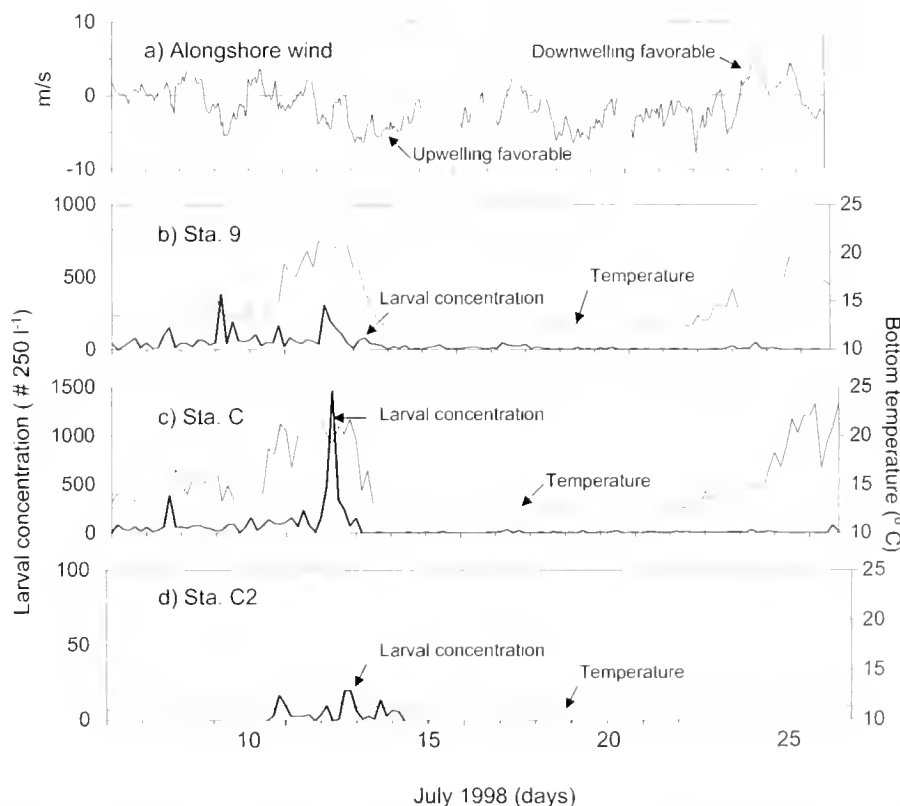


Figure 2. Alongshore winds (a) and surfclam larval concentrations and bottom temperatures at (b) Sta. 9, (c) C, and (d) C2 in July 1998. Alongshore winds are hourly averaged wind intensities (10 m above the ground) from the southwest (negative, upwelling favorable) or from northeast (positive, downwelling favorable). Note scale differences in larval concentrations. Larval data for Sta. C2 were only available for July 11–14.

TABLE 1.

Shell length distribution of surfclams in plankton samples when high concentrations were present (bivalve larvae  $>150\ \mu\text{m}$  were identified). Laboratory studies (Snelgrove et al. 1998) indicate that larvae are ready to settle at shell lengths (SL)  $230\text{--}290\ \mu\text{m}$ , so larvae  $<230\ \mu\text{m}$  SL were probably not competent, and some individuals  $>320\ \mu\text{m}$  SL were likely to be resuspended juveniles.

Station, Date (hours)	$<230\ \mu\text{m}$	$230\text{--}290\ \mu\text{m}$	$290\text{--}320\ \mu\text{m}$	$>320\ \mu\text{m}$	Total
Sta. 9					
July 8 (7:00)	0	29	41	30	100
July 9 (19:00)	1	71	24	4	100
July 10 (3:00)	1	38	19	2	60
July 11 (11:00)	0	70	24	7	100
July 12 (11:00)	0	39	42	1	82
Sta. C					
July 8 (7:00)	15	39	41	5	100
July 10 (15:00)	0	78	20	2	100
July 11 (19:00)	0	74	13	13	100
July 12 (3:00)	18	70	9	3	100
July 12 (19:00)	3	79	17	1	100
July 12 (23:00)	11	67	20	2	100
July 13 (3:00)	13	76	11	0	100
July 13 (7:00)	20	74	4	2	100

Surfclam larval concentrations were relatively high and variable before July 13 at the two inshore stations (Fig. 2). At Sta. C there was a sharp peak in surfclam larval concentration on July 12–13, coinciding with the presence of relatively low salinity wa-

ter (Ma and Grassle 2004). Surfclam larval concentrations were low ( $0\text{--}46\ \text{ind. } 250\ \text{l}^{-1}$ ) inshore after July 13 during the sustained upwelling. At offshore Sta. C2, surfclam larval concentrations were low ( $\leq 20\ \text{larvae } 250\ \text{l}^{-1}$ ) from July 11–14 at the time when high concentrations were found inshore.

Except for the magnitude of the peak in larval surfclam concentration at Sta. C on July 12–13, the integrated larval supplies to Sta. 9 and C during July were very similar. Larvae present in the water column at that time were mostly of a size suggesting that they were competent to settle based on laboratory culture studies (Table 1). In Table 1, shell length distributions for surfclams in plankton samples where they were abundant are shown on 5 d between July 8 and 13. There was a small percentage of probable precompetent larvae ( $<230\ \mu\text{m}$ ), 30–80% competent larvae ( $230\text{--}290\ \mu\text{m}$ ) and a small fraction of juvenile surfclams  $>320\text{--}\mu\text{m}$  shell length that had been resuspended from the bottom. This leaves a sizeable proportion (5–40% in the different samples) ranging in shell length from  $290\text{--}320\ \mu\text{m}$ , which probably included both larvae and recently metamorphosed individuals.

#### Abundances of Initial Surfclam Recruits

At inshore Sta. 9, total surfclam densities were  $>20\ \text{ind. core}^{-1}$  in July and  $<5\ \text{ind. core}^{-1}$  in August and September (Fig. 3). Relatively high surfclam densities appeared again in November with the recruitment of fall-spawned larvae in October and November. This October–November pulse was observed at all three stations. At Sta. C, surfclam densities were very high ( $>200\ \text{ind. core}^{-1}$ ) in July. At offshore Sta. C2, surfclam densities were  $<10$

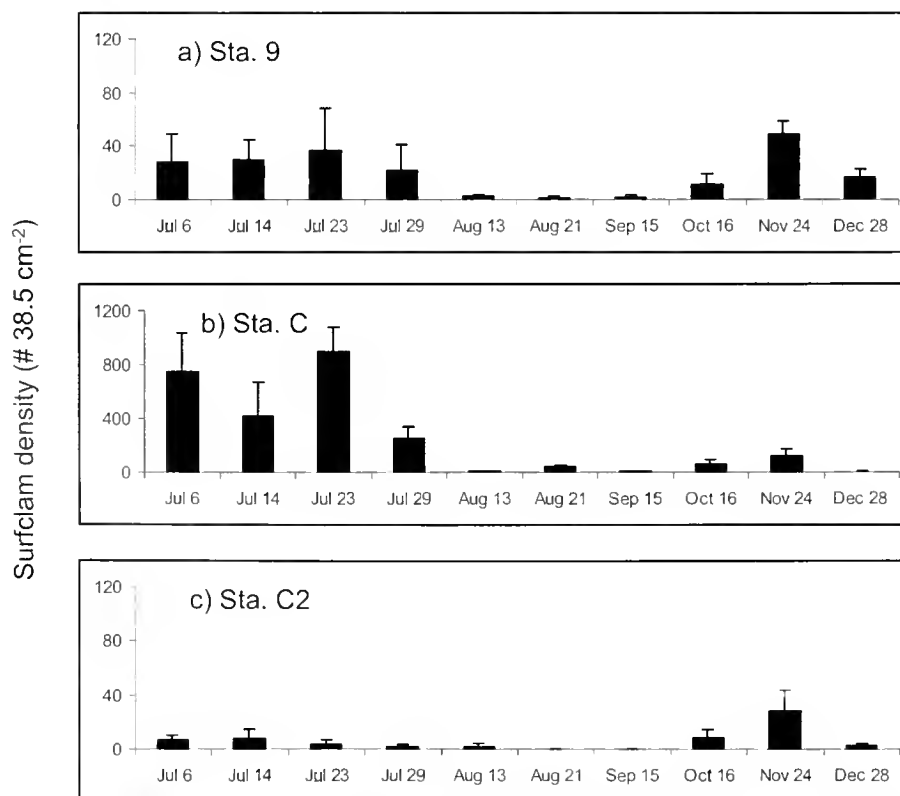


Figure 3. Mean ( $\pm$ SD) number of surfclams  $\text{core}^{-1}$  ( $38.5\ \text{cm}^2$ ) in samples at three LEO-15 stations from July to December 1998 (a) Sta. 9, (b) Sta. C, (c) Sta. C2. Note difference in scales of y-axes with surfclams being an order of magnitude more abundant at Sta. C. Temporal patterns in abundance were similar among the three stations.

TABLE 2.

Analysis of variance of benthic abundances of initial surfclam recruits (<360  $\mu\text{m}$  shell length) on four sampling dates in July 1998 at three LEO-15 stations. Means with common letters not significantly different (with an overall significance level  $\alpha = 0.05$ ).

Sources	DF	Sum of Squares	Mean Square	F value	P
Date	3	13.37	4.46	65.36	0.0001
Station	2	21.59	10.80	158.39	0.0001
Date $\times$ station	6	2.18	0.36	5.33	0.0001
Error	69	4.70	0.068		
<b>Stations</b>	<b>July 6</b>	<b>July 14</b>	<b>July 23</b>	<b>July 29</b>	
Mean comparison within stations					
Sta. 9	1.215 <sup>a</sup>	1.211 <sup>a</sup>	0.750 <sup>b</sup>	0.226 <sup>c</sup>	
Sta. C	2.365 <sup>a</sup>	2.084 <sup>a</sup>	1.146 <sup>b</sup>	0.930 <sup>b</sup>	
Sta. C2	0.613 <sup>a</sup>	0.479 <sup>a</sup>	0.151 <sup>b</sup>	0.050 <sup>b</sup>	
Pooled mean comparison among dates					
	1.398 <sup>a</sup>	1.258 <sup>a</sup>	0.682 <sup>b</sup>	0.402 <sup>c</sup>	

Pooled mean comparison among stations: <sup>a</sup>1.631 (Sta. C); <sup>b</sup>0.850 (Sta. 9); <sup>c</sup>0.323 (Sta. C2).

ind core<sup>-1</sup> throughout the summer and fall and about triple that in November with the fall recruitment (Fig. 3).

Analysis of variance for initial surfclam recruits (referring to individuals with shell lengths <360  $\mu\text{m}$ ) in July showed that sampling date, stations, and the interaction between date and station had significant effects on densities (Table 2, Fig. 4). The pooled initial recruits from the three stations were significantly more abundant on July 6 and July 14. Initial recruitment was highest at Sta. C and lowest at Sta. C2. At Sta. C and C2, the number of initial recruits was significantly higher on July 6 and 14 than on July 23 and July 29 (Table 2). At Sta. 9, initial recruitment was significantly higher on July 6 and 14 and lowest on July 29.

The initial surfclam recruits present in the benthos on July 14 at all three stations were a likely consequence of the competent larvae present in the water column in the previous sampling interval (Fig. 4). Initial surfclam recruitment at each of the stations was roughly proportional to larval supply, with the highest larval supply and recruitment at Sta. C and the lowest at Sta. C2. However, the difference in initial recruitment between Sta. 9 and C was comparatively great ( $\sim 10$  times) compared with larval supplies to the two inshore stations. The average larval surfclam concentrations during July 6–14 were 79 ind. 250 l<sup>-1</sup> at Sta. 9 and 119 ind. 250 l<sup>-1</sup> at Sta. C. Low abundance of initial surfclam recruits in the benthos at all three stations at the end of July was correlated with low larval supply in the last two July sampling intervals.

#### Short-term Growth of Surfclams

Three modes were identified by the C.A.MAN procedure for the surfclams sampled on July 6 at Sta. 9 and C and two modes for the other three dates in July (Fig. 5). Growth rates were calculated when advancement of modes with time could be established (lines shown on Fig. 5). Only modes with weights (proportions) >0.2 (20%) were included for growth estimation (the weights of all modes at one date/station combination add up to 1). Both linear ( $\mu\text{m d}^{-1}$ ) and exponential ( $\text{d}^{-1}$ ) growth rates were calculated. Two estimates were made at Sta. 9 and six at Sta. C. (Fig. 5 and Fig. 6). Only the results using the linear growth model are shown. Linear growth rates for initial surfclam recruits (<360- $\mu\text{m}$  shell length)

ranged from 9.7–18.1  $\mu\text{m d}^{-1}$ . Exponential growth rates for the same individuals ranged from 0.029–0.052  $\text{d}^{-1}$ . Linear growth rates of individuals >360- $\mu\text{m}$  shell length ranged from 25.0–51.6  $\mu\text{m d}^{-1}$  (0.021–0.081  $\text{d}^{-1}$  for exponential growth rates). There were too few surfclam recruits at Sta. C2 to permit an analysis of growth, and they ranged mainly from 260–960  $\mu\text{m}$  shell length in July (more similar to Sta. 9).

The surfclams recruiting at Sta. C in the July 6–14 interval did not appear as a separate mode on July 14 (Fig. 5). The first mode on July 14 at Sta. C was likely composed of survivors from the first mode on July 6 and larvae settling during July 6–14. This could have resulted in an underestimate of the growth rates of surfclams in the first mode on July 6. Visually selecting the shell length with the highest frequency (highest bar in Fig. 5) on July 14 as the "mode" for survivors from July 6 would increase the linear growth-rate estimate by only 4  $\mu\text{m d}^{-1}$ .

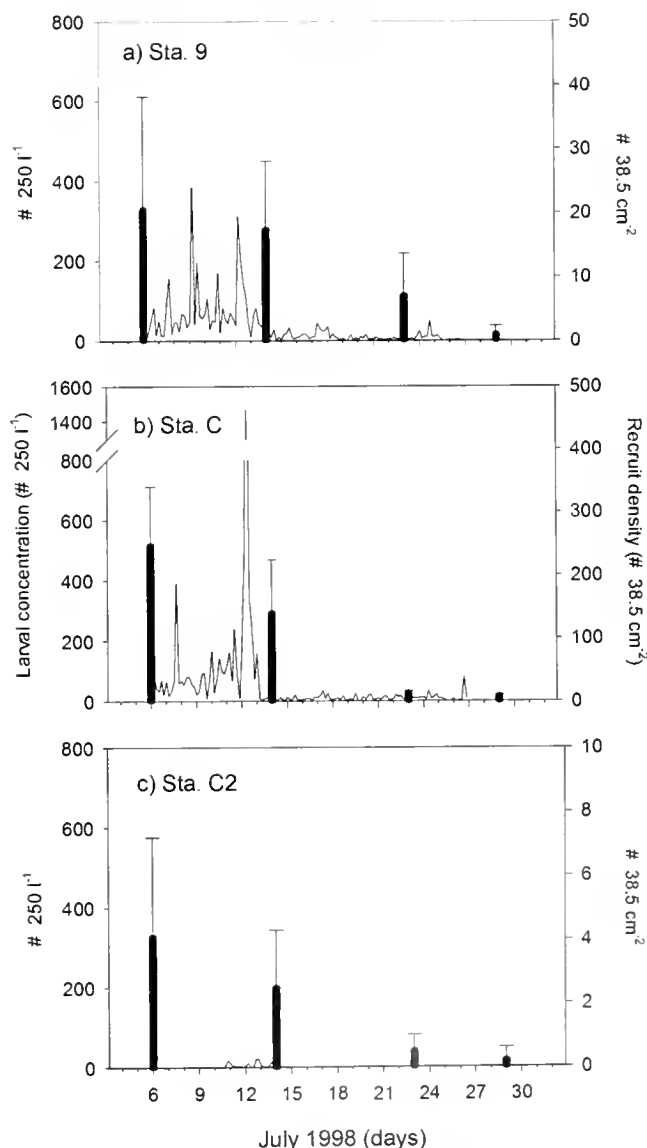


Figure 4. Larval concentration (# 250 l<sup>-1</sup>, lines) and density + SD (# 38.5 cm<sup>-2</sup>, bars) of initial surfclam recruits (<360  $\mu\text{m}$  shell length) in July 1998 (a) Sta. 9, (b) Sta. C, (c) Sta. C2. Note differences in scales on y-axes for surfclam densities in benthic samples and larval data available from July 6–27 at Sta. 9 & C and from July 11–14 at Sta. C2.

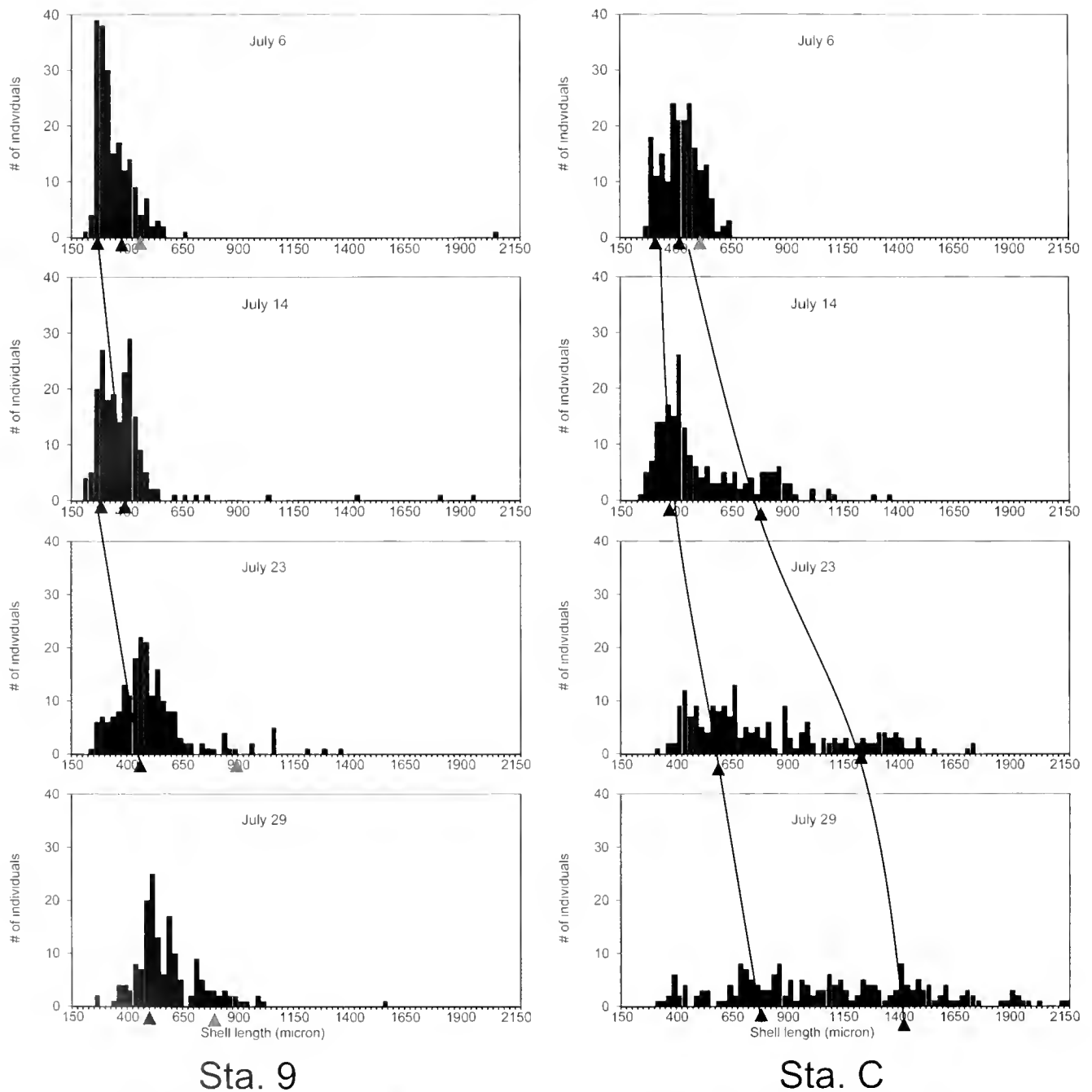


Figure 5. Shell length (SL) distribution of surfclams in July 1998 at Sta. 9 and C ( $n = 200$  for all panels except Sta. 9, July 29 [ $n = 162$ ]). Dark arrows indicate mean SL of cohorts identified by C.A.MAN (Böhning et al. 1992); light arrows have weights  $<0.2$  (total weights are 1.0 on each panel). Lines represent advancements of modes with time. At Sta. 9, advancements of modes were less clear because of lack of accumulation of larger juvenile surfclams, and only two advancements were established. See Figure 2 for temperatures and upwelling/downwelling conditions during July 6–14, July 14–23, and July 23–29.

The intersampling growth periods at Sta. 9 and C in July provided a contrast in temperature regimes: interval 1 (July 6–14) was characterized by temperatures varying from  $13^{\circ}\text{C}$  to  $22^{\circ}\text{C}$ , including some very rapid increases and decreases; interval 2 (July 14–23) was characterized by uniformly low temperatures ( $12^{\circ}\text{C}$  to  $13^{\circ}\text{C}$ ) during upwelling; interval 3 (July 23–29) was more like interval 1, with temperatures varying from  $12^{\circ}\text{C}$  to  $23^{\circ}\text{C}$  at Sta. C and  $13^{\circ}\text{C}$  to  $20^{\circ}\text{C}$  at Sta. 9 (Fig. 1). There appeared to be no relationship between growth rates and the prevailing temperature regimen (Fig. 6).

## DISCUSSION

There was good correspondence between the timing of the highest initial surfclam recruitment and surfclam larval supply at the two inshore stations in July 1998. There was also a rough correlation at all three stations between the magnitude of larval surfclam supply during the period July 6–14 and the abundance of initial surfclam recruits, with Sta. C having the highest larval supply and the highest abundance of recruits and Sta. C2 the least. This relationship between surfclam larval supply and initial re-

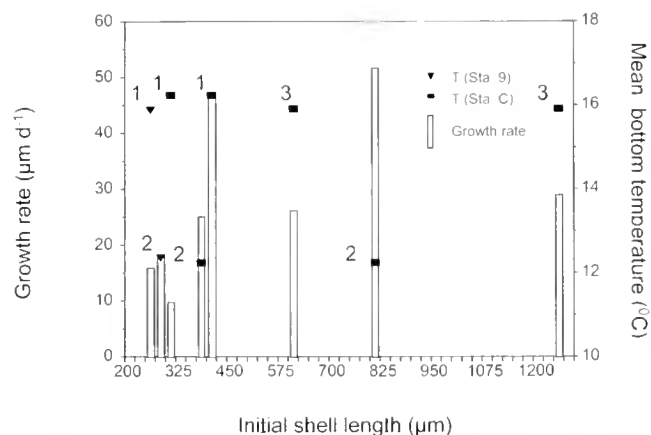


Figure 6. Linear growth rates ( $\mu\text{m d}^{-1}$ , open bars) of surfclams calculated from C.A.MAN analysis of shell length distributions of surfclam juveniles at Sta. 9 and Sta. C on four dates in July. Mean bottom temperatures corresponding with growth-rate estimates during intervals July 6–14, July 14–23, and July 23–29 (marked 1, 2 and 3) are represented with filled triangles for Sta. 9 and filled squares for Sta. C.

cruitment had previously been demonstrated experimentally at the same three stations in 1997 (Ma 2005), although in that year the correlation was significant for Sta. C and C2 but not for Sta. 9. At Sta. 9 the abundance of initial surfclam recruits was less than would have been expected given the similarity in larval supply between Sta. 9 and C, the two inshore stations, which are only one kilometer apart. Ma (2005) has hypothesized that the generally lower initial surfclam recruitment at Sta. 9 compared with Sta. C over several years (Weissberger & Grassle 2003, Quijon & Grassle, in prep.) could be a consequence of stronger near-bottom flows at Sta. 9, especially during storms. These flows could inhibit surfclam larval settlement and/or be responsible for resuspending sediment and winnowing initial recruits out of the sediment and depositing them on the crest and seaward side of Beach Haven Ridge. This hypothesis is partly supported by the difference in sediment grain size between Sta. 9 and C (Craghan 1995), namely coarser sediment characterizes Sta. 9, and it may also explain the great difference ( $\sim$  one order of magnitude) in initial surfclam recruitment between the two inshore stations during downwelling in July 1998 in the face of relatively similar integrated larval supplies.

Based on shell lengths, many of the surfclams present in core samples inshore on July 6 settled in June, probably in association with several upwelling/downwelling periods during that month, namely relaxation or downwelling on June 9–12 and June 17–30. After the initial larval settlement pulses in June and early July, a sharp decline in surfclam densities was observed, and there were few or no individuals remaining in the sediments in August and September. Assuming that there was no new settlement and the decline in density was caused by mortality only, the estimated mortalities ( $z$ ) using an exponential survival equation  $N_{t+\Delta t} = N_t \times e^{-z\Delta t}$  (e.g., Hilborn & Walters 1992) were 0.21–0.22  $\text{d}^{-1}$  from July 23–29 and from July 29 to August 13. The absence of larval settlement was reflected in the very low numbers of surfclams with shell lengths  $<360 \mu\text{m}$  on July 23 (immediately after upwelling), on July 29, and August 13, especially at Sta. C. However, the estimated mortalities could also be affected by resuspension and bedload transport of surfclam recruits to or from other locations.

The mortality of juvenile surfclams was likely caused by multiple impacts of numerous predator species. They include several

species of brachyuran crabs (Stehlik 1993), hermit crabs (Newby 2005), crangonid shrimp (Viscido 1994, Viscido et al. 1997), naticid snails (Weissberger & Grassle 2003, Quijon & Grassle, in prep.) and starfish (Weissberger & Grassle 2003). It is likely that the relative importance of the predators varies from year to year depending on the timing of settlement and initial abundance of the juvenile stages that prey on the smallest surfclams. For example, the 1993 summer year class of surfclams was preyed on by the naticids *Neverita duplicata* and *Euspira heros* and the sea star *Asterias forbesi*. By virtue of the high density of recently settled sea stars beginning in July of that year at Sta. 9 and C, and their high feeding rates, their impacts on juvenile surfclam density were estimated to be much higher ( $4,500 \text{ surfclams m}^{-2} \text{ d}^{-1}$ ) than the impacts of naticid predation ( $90 \text{ surfclams m}^{-2} \text{ d}^{-1}$ ) (Weissberger & Grassle 2003). In 1998 no sea stars were observed in the benthic samples so it is likely that the declining densities in August and September in the present study were a combination of crustacean and naticid predation (Quijon & Grassle, in prep.).

Total surfclam densities increased in October and November at all three stations. More than 80% of individuals in November at the two inshore stations were  $<430 \mu\text{m}$  shell length, and  $>50\%$  were  $<430 \mu\text{m}$  shell length at the offshore station. It is highly likely that these increases in densities were caused by new larval settlement and recruitment in the fall, the result of spawning by surfclams living below the summer thermocline (Tarnowski 1982, Wagner 1984, Weissberger & Grassle 2003, Ma & Grassle 2004). When the thermocline breaks down, warmer water reaches the bottom, inducing the offshore clams to spawn. Pulses of high larval surfclam concentrations were previously observed in the water column in the fall (September to October) at a southern New Jersey site within 80 km of the LEO-15 research area (Haskin et al. 1981, Tarnowski 1982). We also have unpublished data showing that fall-spawned surfclam larvae may persist in the water column at LEO-15 into December and even the following February (Gregg, Tucker, & Grassle unpubl. data), though whether the larvae found in the plankton in February settle and metamorphose successfully is unknown.

Urban (2002) evaluated the applicability of several standard models to the growth of different developmental stages of pearl oysters, including larvae, plantigrades, postlarvae, and juveniles. Like previous authors he concluded that the larvae and plantigrades grow linearly or exponentially (e.g., Bayne 1965, Beaumont & Budd 1982, Rose & Baker 1994). The linear growth rates for initial surfclam recruits ( $<360 \mu\text{m}$ ) in our study were 9.7–18.1  $\mu\text{m d}^{-1}$ . Subsequent growth rates of juveniles  $>360 \mu\text{m}$  shell length were somewhat higher (25.0–51.6  $\mu\text{m d}^{-1}$ ). These growth rates were comparable to those estimated by Weissberger and Grassle (2003) for clams settling in similar densities in 1993 (14.6–63.5  $\mu\text{m d}^{-1}$ ). No clear trend of increased linear growth rate with increased initial size (an indication of exponential growth) was observed in our study. The lower growth rates for the initial surfclam recruits ( $<360 \mu\text{m}$ ) may be a result of the period of time between loss of the larval velum and formation of labial palps at metamorphosis and development of functional suspension-feeding structures (fused siphons and gills). This has not been examined directly in *Spisula solidissima*, but in other bivalves these developmental processes may take one to several days depending on temperature (e.g., Bayne 1971, Reid et al. 1992).

Plotting 1993 growth rates directly against bottom temperature, Weissberger and Grassle (2003) found some correlation up until September. In the present study there was no evidence of any growth rate/temperature correlation for the smallest clams in July

1998. Of course many factors may affect the growth of bivalve recruits, not just temperature. They include population density and the availability of appropriate food. At different intertidal sites in Maine, surfclam juveniles grew faster at sites with warmer water temperatures and higher levels of chlorophyll *a* (Davis et al. 1997). Because upwelling at the LEO-15 sites is associated with high POC and chlorophyll *a* values (Glenn et al. 2004), it is possible that growth of recently settled surfclams during the July upwelling (July 14–23) was enhanced by an increased flux of food particles to the bottom, thus counterbalancing the negative effects of low prevailing temperatures on growth. The LEO-15 research area also represents one of the recurrent hypoxic nodes associated with summer upwelling along the New Jersey coast, but the potential effects of high variance in bottom water O<sub>2</sub> concentrations during July (Boehme et al. 1998) on both juvenile surfclam growth and mortality have not been studied.

Other studies that have examined early growth of surfclams have generally been conducted over much longer time intervals and in some cases under relatively nonnatural field conditions (e.g., Belding 1910, Starypan 1976, Jones et al. 1978), and the initial lengths of the surfclams were at least several millimeters. In a study of surfclams using the growth bands laid down in the shell, Chintala and Grassle (2001) found that surfclams in the inner shelf environment off New Jersey generally reached shell lengths of 10.3–22.0 mm when the first band was laid down, with no significant differences in mean shell length among nine nearshore zones. Although these shell lengths are considerably less than in some other studies (e.g., Jones et al. 1983), they are consistent with previous estimates for the LEO-15 area, namely that surfclams settling in July 1993 reached a maximum shell length of 16.0 mm by December (Weissberger & Grassle 2003).

One potential complication for field studies of bivalve growth rates using modal length frequency analyses is that size-selective predation may effectively remove particular size classes. There was some suggestion that naticid predators could have selectively removed surfclams 2–5 mm in shell length during the late summer and fall in 1993, and that *Asterias forbesi* might have targeted

clams 5–12 mm in length in October and November of the same year (Weissberger & Grassle 2003). The effects of size-selection by small crustacean predators of surfclams in the LEO-15 research area remain largely unstudied. In late July 1998, there were many fewer surfclams >1,000- $\mu$ m shell length at Sta. 9 than at Sta. C (Fig. 5). This may be an example of initial predator swamping at Sta. C where juvenile surfclams were an order of magnitude more abundant than at Sta. 9 in early July. This would allow some individuals to find a size refuge from predators preying selectively on the smallest individuals.

This study has provided further evidence of the close relationship between upwelling/downwelling events in June and July with surfclam larval supply and initial recruitment in June and July in the inner shelf environment. In addition, the study has provided realistic field estimates of early growth rates under both upwelling and downwelling conditions. Analyses of length frequency distributions and abundances of juvenile surfclams have provided further estimates of the limits on early growth in the nearshore environment and of the probable impacts of the many surfclam predators. Such knowledge is important to the understanding of surfclam recruitment processes and to management of the surfclam fishery in the Mid-Atlantic Bight. Potentially, this kind of information can have implications for shellfish species in other geographic regions.

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## VARIATIONS IN GROWTH AND REPRODUCTION OF BAY SCALLOPS (*ARGOPECTEN IRRADIANS*) (LAMARK, 1819) FROM SIX SUBPOPULATIONS IN THE NORTHEASTERN GULF OF MEXICO

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**ABSTRACT** Bay scallops, *Argopecten irradians*, sustained a valuable commercial and recreational fishery in Florida during the middle part of the last century. In 1994, after decades of declining stocks, state managers closed this fishery to commercial harvest and severely limited the recreational harvest. In Florida, most bay scallops reproduce only once, generally during the fall at an age of roughly 12. The current 10-week recreational harvest season, July 1 through September 10, occurs at a time when somatic and reproductive tissues are changing rapidly in preparation for the fall spawning season. This study describes changes in tissue weights (reproductive, somatic, visceral) during the 2002 harvest season in scallops collected from six subpopulations along Florida's Gulf of Mexico coast. We observed tissue-specific patterns of weight change during the 7-month study and also noted regional differences. In three Panhandle sites (St. Joseph Bay, Lanark Village, and St. Marks), a shift from somatic growth to reproductive growth occurred later in the year and was more intense than in peninsular sites (Steinhatchee, Homosassa and Anclote). We also monitored recruitment of juvenile scallops at three of the sites from July of 2001 through June of 2003. There were protracted fall and winter peaks within a background of constant, low-level recruitment. The harvest limits allow each person to collect two gallons (7.55 L) of whole scallops or one pint (0.47 L) of adductor muscle meat each day. In June, just prior to the harvest season, the number of whole scallops that would be collected varied significantly between sites (range 55–203), as did the equivalent yield of adductor muscles (range 143–342). Muscle size peaked in August or September, depending on location. The allowable number of scallops collected within the volume-based limits had decreased (41–112 whole scallops or 84–116 shucked scallops) and between-site variability in the numerical harvest was lower.

**KEY WORDS:** *Argopecten irradians*, Florida, gonadal-somatic index, growth, harvest, recruitment, bay scallop

### INTRODUCTION

Bay scallops, *Argopecten irradians* (Lamarck, 1819), were once a valuable commercial fisheries species for the state of Florida. During the 1950s, harvests commonly exceeded 100,000 pounds per year of adductor muscle (Murdock 1955) and peaked at 401,283 pounds in 1958 (Rosen 1959). There is also evidence for prehistoric sustenance harvest (Marelli & Arnold 2001) and a modern cultural heritage of recreational harvest. Stocks declined during the 1960s and 1970s, with a concomitant decrease in commercial harvest to levels between 10,000 and 20,000 pounds of adductor muscle per year by the 1980s (for example, see Joyce 1982). The abundance of scallops in many subpopulations declined to very low densities or disappeared completely. This decline was first documented in Pine Island Sound, then in Tampa Bay, the Anclote River Estuary and finally in the Homosassa River region. Similar declines have been observed in the western Florida Panhandle (Arnold et al. 2005).

In response to the decline of bay scallop abundance in Florida waters, harvest restrictions were instituted in 1985 and expanded in 1994. Management of the fishery targeted both commercial and recreational harvest. Commercial harvest was eliminated and recreational harvest was severely curtailed in 1994. All subpopulations south of the Suwannee River were closed to recreational harvest. The daily harvest limit of five gallons (18.88 L) of whole scallops per person was reduced to the current limit of two gallons (7.55 L) of whole scallops or one pint (0.47 L) of shucked adductor muscle per individual. The duration of the harvest season was reduced from 10 mo per year to 10 wk per year. Based on a survey program that was initiated in 1993, subpopulations are currently grouped into 3 classes: healthy (average density >25 scallops/600 m<sup>2</sup>), transitional (5–25 scallops/600 m<sup>2</sup>) or collapsed (<5 scallops/

600 m<sup>2</sup>). In 1994, all populations south of the Suwannee could be classified as either collapsed or transitional. To accompany the management actions, active restoration was initiated in 1998 and continues to this day. The goal of the restoration program was not to produce scallops for direct harvest but to create high-density spawning aggregations that may then seed the natural populations. Hatchery-reared scallops are held in cages at densities up to 300 per 0.6 m<sup>2</sup> in Crystal Bay, Homosassa River estuary, the Anclote River estuary (hereafter Homosassa and Anclote), Tampa Bay and Sarasota Bay. Subsequent increases in scallop abundance in the Homosassa subpopulation (Arnold et al. 2005) have been extensive enough that the recreational fishery was reopened in 2002.

In Florida, recreational harvest occurs during summer to accommodate the most popular means of collection, snorkeling. Current harvest of bay scallops in Florida is concentrated on a limited number of traditional subpopulations. Although the harvest season occurs immediately prior to the spawning season, previous research indicates that harvest occurs when muscle yield per individual is maximal (Barber & Blake 1983). A similar management approach is applied in the northeastern United States (bay scallop harvest is timed to maximize the yield of adductor muscle; Rhodes 1991, Estabrooks 2003). However, those animals are allowed to pass through their first spawning season and are harvested at a time when adductor muscles have begun a period of new growth, when most animals in the population are roughly 15 mo of age.

Bay scallops have been described as quasi-semelparous: most individuals spawn only once, but a small portion survive for one or more additional spawns (Orensanz et al. 1991). The traditional view of this species suggests that local subpopulations will spawn synchronously, either during warming spring or cooling fall periods (Sastry 1963, 1970). Recent research suggests that this is not always the case in Florida subpopulations. Arnold et al. (1998) monitored recruitment of spat in four distinct subpopulations on Florida's west coast from early August through late February. They found that whereas distinct peaks occurred in each locale,

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some recruitment occurred throughout the study in three of the four subpopulations. In a separate study, Bologna (1998) collected recruits in 12 of 28 mo during 1994 through 1996 in St. Joseph Bay, Florida. Over the course of the study, recruits were collected in 8 of the 12 calendar months. Secondary recruitment periods have also been observed in Long Island, New York (Tuttlebach et al. 1999).

Tissue growth in bay scallops is most rapid during the first year of life. Somatic growth will often precede gonadal growth, with energy stored in the adductor muscle as glycogen and protein and in the digestive gland as lipid (Barber & Blake 1981). Growth of these two organs may slow or be negative once gametogenesis begins, at which point gonad growth is very rapid (Bricelj et al. 1987, Barber & Blake 1983). Postspawn Florida scallops can suffer high mortality that is correlated with depleted energy reserves (Barber & Blake 1983). In northeastern United States populations, a higher percentage of scallops survive for additional spawns (Bricelj 1992). The scallops that survive a spawning cycle will initiate new growth in the adductor muscle, and gonadal-somatic indices do not attain as high a level during subsequent spawns, although actual gonad weight continues to increase (Bricelj 1992).

In this study, we explore small-scale variations in tissue-specific size and growth during a single harvest season (2002) in six subpopulations of bay scallops along Florida's Gulf of Mexico coast. The results from monitoring juvenile scallop recruitment are used to refine the interpretation of tissue growth and reproduction in three of those six subpopulations. The changes in the amount of allowable harvest, in terms of the number of scallops harvested, that accompany the changes in scallop shell and tissue (in particular adductor muscle) volume are described.

## MATERIALS AND METHODS

### Adult Collections

*Argopecten irradians* were collected from six sites along the Florida Gulf coast between Port St. Joe and Tampa Bay ( $n = 1226$ , Fig. 1). Four sites (Anclote, Homosassa, Steinhatchee and St. Marks) are part of the Big Bend region of Florida, a zero-energy coastline. The coastal seagrass bed found here varies between 11 and 35 km wide, an area approximately 3,000 km<sup>2</sup>, and is dominated by *Thalassia testudinum*, *Syringodium filiforme* and *Halodule wrightii* (Iverson & Bittaker 1986). The St. Joseph Bay and Lanark sites are also low energy and have similar seagrass communities but are protected by barrier islands.

The bay scallop abundance at four of the six sites is routinely monitored (Arnold et al. 2005). Anclote was the southernmost site. During 11 y of survey there (1994–2004), the average scallop density varied between 0.15 and 47.35/600 m<sup>2</sup>. During three of the years the density was considered healthy, during four years it was considered transitional, and during four years it was considered collapsed. It has not been open for harvest since 1993. Over the same period, the Homosassa population was considered healthy in 5 years, transitional in 3 years, and collapsed in 3 years. The study year, 2002, was the first year this site had been opened to harvest since 1993. Steinhatchee is the most stable subpopulation in the state; its density had been considered healthy in all years except 2004, when it was classified as transitional. The St. Marks and Lanark subpopulation densities are not monitored. The St. Joseph Bay subpopulation was also generally healthy (7 of the 11 y), although it was classified as transitional in 2 years and collapsed in 2 years. All four monitored subpopulations were considered to be healthy during 2002.

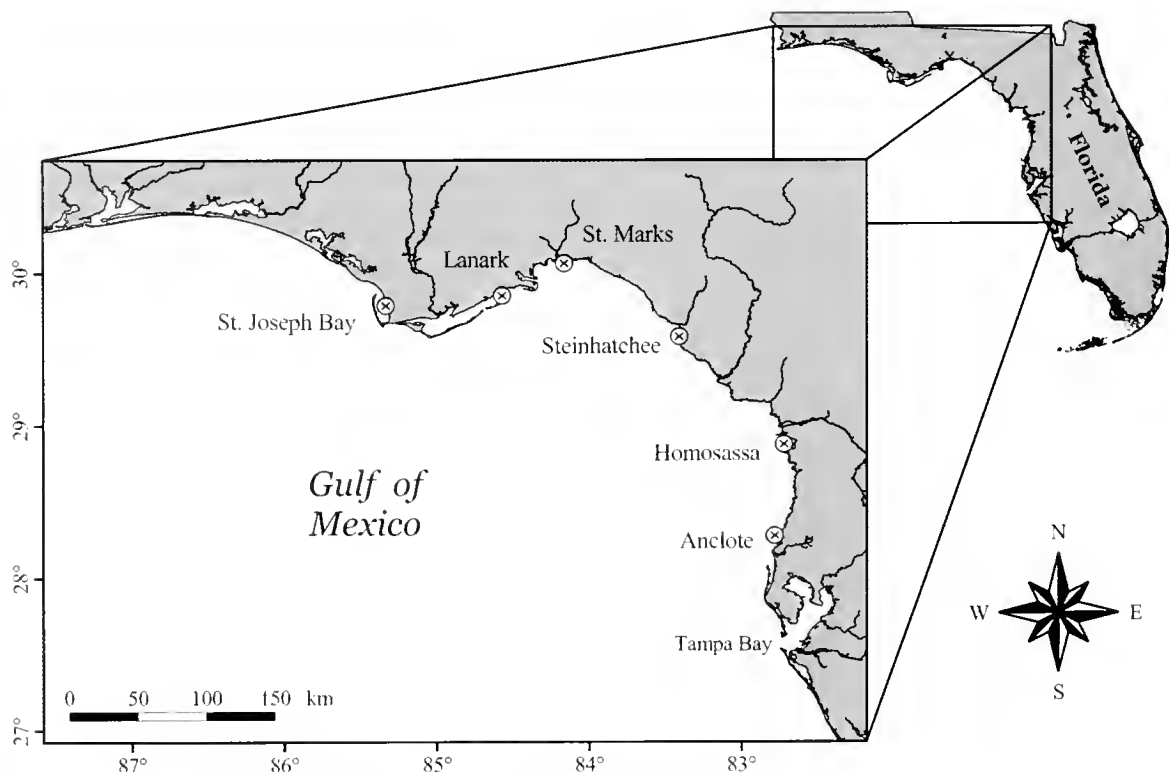


Figure 1. Locations of the six sampled subpopulations of Florida bay scallops, *Argopecten irradians*.

Our goal was to collect samples of 30 adults from each study site at 3-week intervals between May and September 2002 (Table 1), with at least one sample set collected before and one sample set collected after the harvest season. However, we began collecting samples as early as April 24 at Anclote and Homosassa and May 16 to May 17 at Steinhatchee, Lanark and St. Joseph Bay (because collections were made opportunistically). The study was terminated as early as September 3 at Steinhatchee (because of logistic constraints) and as late as October 10 at the Homosassa site (Table 1). Live scallops were collected opportunistically by hand and held in coolers for transport to shore. Samples collected at the Anclote and Homosassa sites were placed directly into freezers at the Fish & Wildlife Research Institute (FWRI) in St. Petersburg. Samples collected at all other locations were initially frozen and then were shipped overnight (packaged with dry ice) to St. Petersburg. All samples were then held at  $-80^{\circ}\text{C}$  until analysis.

#### Shell and Tissue Analysis

Scallops were thawed for at least 1 hour before dissection. Many shells were covered in epibionts such as oysters, jingle shells and slippers snails. These fouling organisms were removed. The soft tissues of the scallop were separated into three components: gonad, adductor muscle, and all other tissue (hereafter termed viscera). Each of the tissues was placed into tared aluminum trays and the wet weights were measured to the nearest 0.1 mg. The volume of the adductor muscle was determined by submerging the muscle in a known volume of water in a graduated cylinder and observing the increase in volume to the nearest 0.2 mL. All components were then dried to constant weight in an oven at  $60^{\circ}\text{C}$  to  $65^{\circ}\text{C}$  for 20–24 h and the dry weights of each of the three tissues measured. Only mature specimens with a gonad dry weight  $<0.001$  g were included in statistical comparisons.

After all tissue was removed, the valves of each scallop were rearticulated and the height, width and length were measured to the nearest 0.1 mm using digital calipers. To estimate the volume of a collection of whole scallops, rearticulated shells were taped back together and the entire sample from each sampling date and location was placed inside a 5,000-mL graduated beaker. The dry volume of the entire sample was estimated to the nearest 100 mL.

This process was repeated three times and an average volume was calculated to estimate the error variance associated with the packing of the shells.

The uneven sampling effort resulted in a matrix of sample dates and locations that was not amenable to a full factorial design. Initial analysis of the data was conducted using an analysis of covariance (ANCOVA), with the variable of interest as the dependent factor (shell height, adductor muscle dry weight, muscle volume, or gonadal-somatic index [GSI]), site as a categorical factor, and Julian date as a covariate. We calculated GSI as the ratio of reproductive tissue to all other tissue (muscle and viscera). When the ANCOVA model showed that the parameter of interest varied significantly either temporally or between sites, further analysis was conducted. We used either Dunn multiple comparison test or Tukey multiple comparison test for uneven sample sizes to identify differences between collection dates within each site or between sites within each month. We calculated growth rate for each sub-population by least squares linear regression, using shell height as the dependent variable and Julian calendar day as the independent variable. In each of the six regressions the residuals were normally distributed and homoscedastic. The slope of the resulting equation can be interpreted as the growth rate in mm per day.

#### Recruit Monitoring

As part of an ongoing study (Arnold et al. 1998, 2005) recruit collectors were deployed in 3 of our study sites: Anclote, Homosassa and St. Joseph Bay. Each recruit collector consisted of a 4-mm mesh, 1/2-bushel citrus bag containing a  $30 \times 48$ -cm panel of 4-mm mesh polypropylene (Ambrose et al. 1992). The collector was attached 0.25 m above a cinder block anchor with 1/2-inch polypropylene rope. A small plastic donut float was attached to the opposite end of the trap, and a crab-trap float was attached to the distal end of the rope, each helping to keep the trap in a roughly vertical orientation off the bottom.

Each trap was placed in seagrass beds at depths of 0.5–1.5 m of water at low tide. Twenty-four collectors were deployed at each site, composed of 2 sets of 12, paired stations. At each station, one trap from each pair was pulled on alternate, 3-week target schedules, so that each trap soaked for roughly 6 weeks (median = 43

TABLE 1.

Summary of collection dates and sample size of bay scallops (*Argopecten irradians*) during 2002. The six collection locations are listed in the first column, and total sample sizes,  $n$ , for each location are shown in the last column. Individual collection dates and sample sizes for each date (given in parentheses) are listed by month.

Location	April	May	June	July	August	September	October	Total
Anclote	4/24 (28)	5/08 (19) 5/30 (29)		7/01 (29) 7/16 (30)	8/09 (33) 8/29 (30)	9/17 (30)	10/08 (32)	260
Homosassa	4/24 (30)	5/08 (34) 5/30 (30)	6/21 (23)	7/16 (30)	8/12 (30) 8/29 (30)	9/17 (30)	10/10 (30)	267
Steinhatchee		5/17 (29)	6/20 (30)	7/10 (30) 7/17 (30) 7/31 (30)	8/13 (30)	9/03 (30)		209
St. Marks			6/25 (33)	7/15 (30)	8/05 (30) 8/27 (33)	9/20 (30)		156
Lanark		5/16 (30)	6/05 (30)	7/09 (30) 7/31 (30)	8/22 (30)	9/12 (30)		180
St. Joseph Bay		5/17 (05) 5/26 (28) 5/31 (10)	6/12 (29)	7/17 (32)	8/15 (30)	9/19 (30)		154

days). Actual soak times varied between 36 and 84 days. Each trap was retrieved and placed in a labeled garbage bag without other preservation and was examined at a later date for scallop recruits. All surfaces (inside and outside of the citrus bag, and both sides of the polypropylene mesh) were examined with the naked eye, and all spat were removed and counted. Recruitment rate was calculated as the number of recruits divided by the number of days the trap had soaked. Mean recruitment rates were calculated weekly for each station by averaging the recruitment rate of all collectors at each location. Results are presented for traps retrieved during the period of July 2001 to June 2003.

## RESULTS

Initial analyses using ANCOVA showed that each of the 4 variables of interest (shell height, adductor muscle dry weight, adductor muscle volume and the ratio of gonadal-somatic index) varied both between sites and temporally (Table 2). In each case the interaction term was also significant, indicating that the differences between the six sites were not consistent during the 6 months of the study. Further analyses were focused on elucidating where these differences existed.

The overall trend was for shell height to increase at each site during the course of the study but at rates that varied between sites. The growth rate ranged from 0.075 mm day<sup>-1</sup> at Lanark Village to 0.208 mm day<sup>-1</sup> at St. Marks (Table 3). Immediately prior to the July 1st opening of the recreational harvest season, mean shell heights at Anclote and Lanark were significantly larger than they were at the other four sites. During the middle of the harvest season (late July and early August) mean shell height had increased slightly and was uniform across the sample region, with the exception of one sample period (August 9–29), when the scallops at St. Joseph Bay were smaller than scallops at all other sites (Table 3). At the conclusion of the harvest season, shell heights at most sites had continued to increase slightly and were similar at most sites. The exceptions were the scallops at St. Marks, which had grown significantly larger than those at all other sites.

Adductor-muscle dry weight and volume both changed in similar ways during the course of the study, exhibiting two general trends. At St. Joseph Bay, adductor muscle volume increased throughout the season. At the other locations, muscle volume peaked in August then declined in September and October (Fig. 2). Adductor-muscle dry weight peaked in August and September at

TABLE 3.

Observed growth rates in bay scallops (*Argopecten irradians*). The growth rate (GR) of the shell height (mm day<sup>-1</sup>) for each subpopulation, and coefficient of determination ( $r^2$ ) for the least squares linear regression are given. Each regression equation was significant at  $P < 0.001$ . Sample sizes are the same as the total sample size in Table 1. Mean shell heights are given for the period immediately prior to harvest season (6/5 to 6/26; except Anclote, where samples were collected on the first day of the harvest season, 7/1), during mid-season (8/4 to 8/29), and after the harvest season had ended (9/12 to 9/20; except Steinhatchee, where the last collection occurred on 9/3, seven days prior to the end of the harvest season). Similar groups, as determined by Tukey multiple comparison test for unequal sample sizes, are indicated by the same letter.

	GR	$r^2$	Pre-season	Mid-season	Post-season
Anclote	0.090	0.4676	54.3 <sup>a</sup>	60.1 <sup>a</sup>	59.0 <sup>b</sup>
Homosassa	0.087	0.4897	45.8 <sup>b</sup>	57.3 <sup>a</sup>	58.9 <sup>b</sup>
Steinhatchee	0.132	0.5023	43.8 <sup>b</sup>	57.7 <sup>a</sup>	55.9 <sup>b</sup>
St. Marks	0.207	0.5663	43.1 <sup>b</sup>	56.2 <sup>a</sup>	63.3 <sup>a</sup>
Lanark Village	0.075	0.4056	54.1 <sup>a</sup>	59.6 <sup>a</sup>	60.7 <sup>b</sup>
St. Joseph Bay	0.135	0.6301	45.7 <sup>b</sup>	54.5 <sup>b</sup>	57.8 <sup>b</sup>

St. Marks and St. Joseph Bay. At the other four sample locations, August peaks were followed by declines in September and October (Fig. 3).

Gonad dry weight also changed over the course of the study, again having two general trends. At the peninsular locations (Anclote, Homosassa and Steinhatchee) gonad dry-weight initially increased slowly then leveled off in August and September. In Panhandle sites (St. Marks, Lanark and St. Joseph Bay), gonad dry weight also initially increased slowly but then increased rapidly in September to a much greater extent than at the three peninsular sites (Fig. 4).

Changes in gonadal-somatic index (GSI) followed two distinct trends. GSI at all sites was low when the study was initiated; mean values ranged from 0.02–0.05 during April through June (Fig. 5). At the three peninsular sites, significant increases in GSI were observed in August and September, but the mean GSI never exceeded 0.12. At the three Panhandle sites, the increases in GSI that

TABLE 2.

Analysis of covariance results from analyses of site-specific and temporal variation in four measures of growth and reproduction in bay scallops (*Argopecten irradians*) during 2002: shell height, adductor muscle dry weight, muscle volume, and the ratio of reproductive to somatic tissue. For each variable, there was one degree of freedom for each of the model and date components, five degrees of freedom for both the by-site comparisons and interaction terms, and 1,214 degrees of freedom for the error term. The mean square and F value for ANCOVA results for each regression term are given. All  $P$  values were  $<0.01$ .

		Error	Intercept	Site	Date	Site × Date Interaction
Shell height	MS	23.26	25177.70	539.65	25779.99	538.68
	F		1082.60	23.20	1108.49	23.16
Adductor dry weight	MS	0.12	30.39	1.03	30.71	1.03
	F		250.78	8.47	253.49	8.51
Muscle volume	MS	1.34	531.23	14.00	536.62	14.06
	F		392.62	10.40	398.62	10.44
Gonad:muscle ratio	MS	0.0011	1.0101	0.0989	1.0147	0.0990
	F		892.88	87.41	897.01	87.50

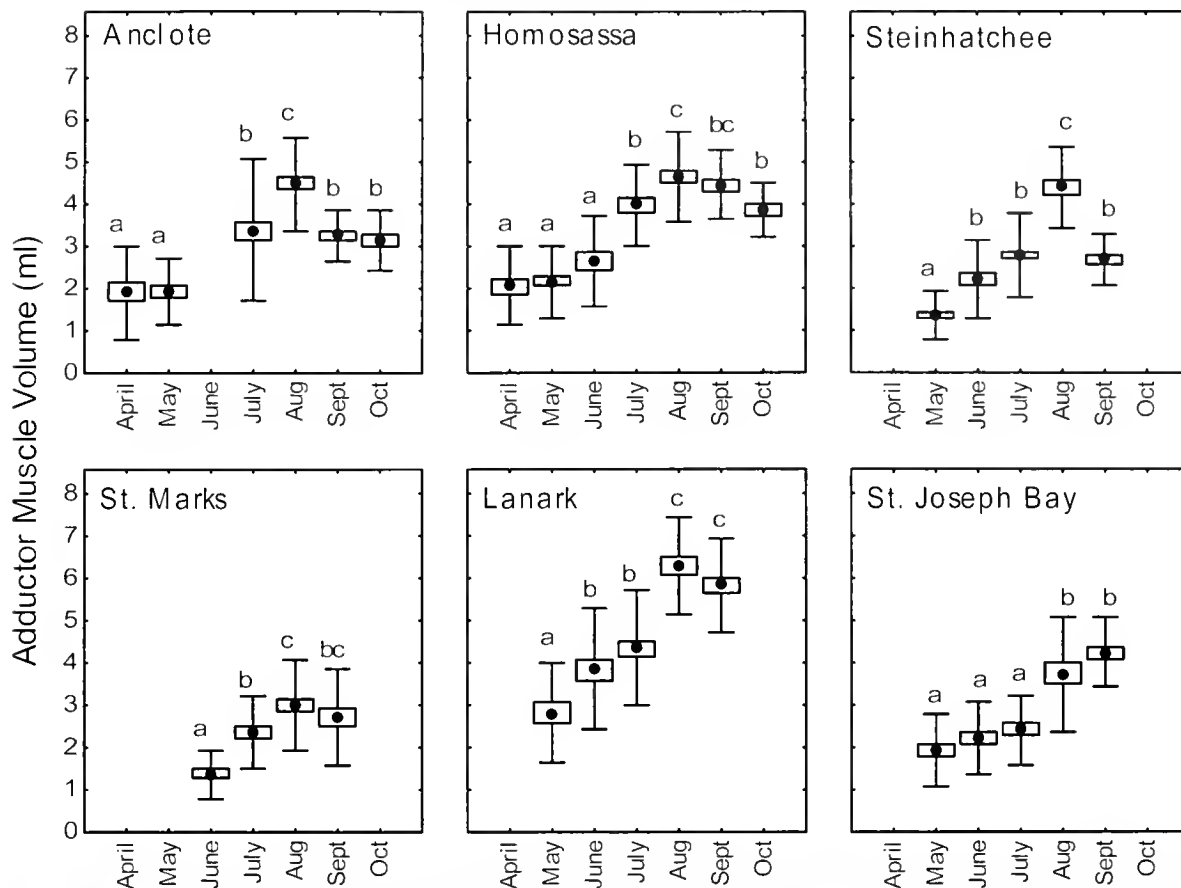


Figure 2. Mean volume of adductor muscles of bay scallops, *Argopecten irradians*, from six subpopulations along Florida's Gulf of Mexico coast. Midpoint, mean; box, standard error; bars, standard deviation. Groups that are not significantly different are indicated by the same letter(s).

occurred in September were more pronounced. Mean GSI in September increased to values of 0.20 in St. Marks, 0.14 in Lanark and 0.19 in St. Joseph Bay.

Three trends in the number of scallops estimated to be harvested by someone collecting their recreational limit were observed. First, the number of scallops that can be legally harvested generally declines as the season progresses, regardless of which measure is used (Fig. 6). This trend was not entirely linear, and an increase in the number of scallops harvested using the one-pint limit did occur late in the season because muscle size began to decline. Second, more scallops could be legally harvested by someone shucking their scallops at sea and collecting one pint of meat than by someone collecting two gallons of whole scallops and shucking them on shore. This pattern is especially true early in the season, but the disparity declines as the season progresses. Finally, during the harvest season Lanark had the largest scallops with the largest adductor muscles, so a fisher harvesting their legal limit from this area would take fewer scallops than at any other area.

If the harvest season were to open in May, a legal harvest would range from 65–215 whole scallops and between 213 and 429 shucked scallops (Fig. 6). Under current regulations, when the season opens on July 1, legal harvest would range from 55–203 whole scallops or 143–342 shucked scallops. When scallops at most sites achieved peak muscle size during early August (midway through the harvest season), legal harvests ranged from 41–112 whole scallops or 84–116 shucked scallops. Thus, there is a dis-

parity between sites in the number of scallops that could be legally harvested, but this disparity declines as the season progresses.

In each year, all three monitored subpopulations had recruitment that occurred with peaks but also with protracted periods of very low levels of recruitment (Fig. 7). At Anclote, spat settled over a protracted period in both years. Recruitment peaked from late October 2001 through January 2002, but some recruits were collected in every sample period from August 2001 through May 2003. Modest peaks also occurred in August and during October through December 2002. At the Homosassa site, spat recruited to collectors from August through June, with peaks in September 2001 through January 2002 and again in August through November of 2002. In St. Joseph Bay, we observed recruitment throughout the study. A large peak in recruitment began in October 2001 and continued through January 2002. A more modest peak in recruitment was observed in late October and continuing through November in 2002. One clear trend is that Homosassa traps (maximum rate of 0.38 spat per bag per day) collected scallops at a rate roughly one order of magnitude less than Anclote traps did (maximum rate of 6.3 spat per bag per day), and Anclote traps collected scallops at a rate roughly one order of magnitude less than traps in St. Joseph Bay did (maximum rate of 48 per bag per day).

## DISCUSSION

In the six subpopulations of bay scallops that we sampled from Florida waters, we observed higher than expected variability be-

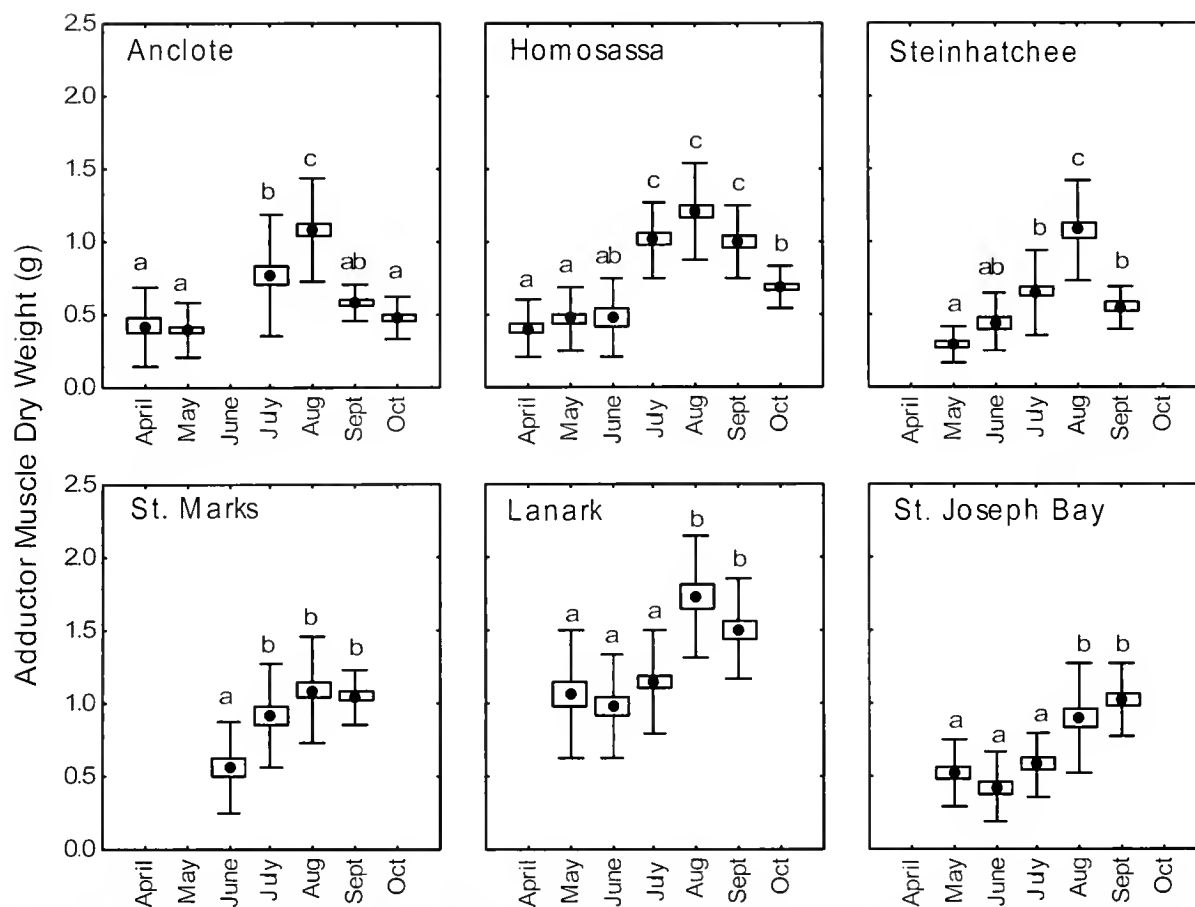


Figure 3. Mean dry weight of adductor muscles of bay scallops, *Argopecten irradians*, from six subpopulations along Florida's Gulf of Mexico coast. Midpoint, mean; box, standard error; bars, standard deviation. Groups that are not significantly different are indicated by the same letter(s).

tween sites and predictable but still variable temporal changes. In each subpopulation, we documented growth and development towards a maximal spawning condition in early fall, as predicted by previous studies (Barber & Blake 1983). All six subpopulations showed a positive trend in shell height and gonad weight, and all of the subpopulations experienced a relative increase in mean muscle weight and volume during the initial portion of the study. However, there were regional differences in the amount of energy devoted to somatic growth versus gonad growth and reproduction. The scallops from the Florida Panhandle sites had more pronounced increases in gonadal-somatic index in September than did scallops from the peninsular sites.

Changes in the value of a ratio can occur when either of its components change but can also remain the same when both components change similarly. In this study, increases in GSI might be expected at the peninsular sites, because scallops at all three had decreases in muscle weight and volume in the fall. However, gonad weight also declined (indicative of a spawning event), so the ratio changed little. These observations do not support the expectation that energy reserves were transferred from muscle to gonad. In two of the three Panhandle sites (Lanark and St. Marks), scallop muscle weight declined, whereas gonad weight increased, resulting in significant increases in GSI, evidence that would support the expectation of a transfer of energy from muscle to gonad. These two subpopulations are most similar to scallops observed by Sastry (1966) and Barber and Blake (1985) in which energy reserves in muscle and digestive gland were transferred into reproductive tissue.

In St. Joseph Bay, both muscle and gonad weights increased, whereas GSI increased greatly. Thus, a contrast between scallops in the two regions suggests that in 2002, those at peninsular sites had limited energy available for an early fall spawn, whereas those at Panhandle sites had relatively more energy for a late fall spawn, as seems to be evidenced by the actual size of the gonad or the GSI.

Studies of recruitment can supply evidence supporting the assumption that peaks in gonad weight and GSI are correlated with a successful spawning event. Settlement of scallops on spat collectors occurred contemporaneously with peaks in GSI (this study). At Anclote, spat settled over a protracted period (August 2002 through January 2003). The initiation of this recruitment was concurrent with the weak peak in GSI. Similarly, at the Homosassa site, a weak peak in GSI was observed in August, and again, spat recruited to collectors in August through December. In St. Joseph Bay, before we discontinued this study, we observed a peak in GSI in September. A large peak in recruitment began in November 2002 and continued through January of 2003, suggesting that the large energetic reserves we observed translated into a large recruitment event in St. Joseph Bay.

Bologna (1998) suggested that the St. Joseph Bay subpopulation spawned year-round but that the peak of the spawning season varied from year to year. In that study, gonadal-somatic condition peaked during the winter, but recruits were collected in nearly every month. Barber & Blake (1981, 1983) found that the scallops in the Anclote estuary devoted most of their assimilated energy to somatic growth in spring. Histological analysis indicated that oo-

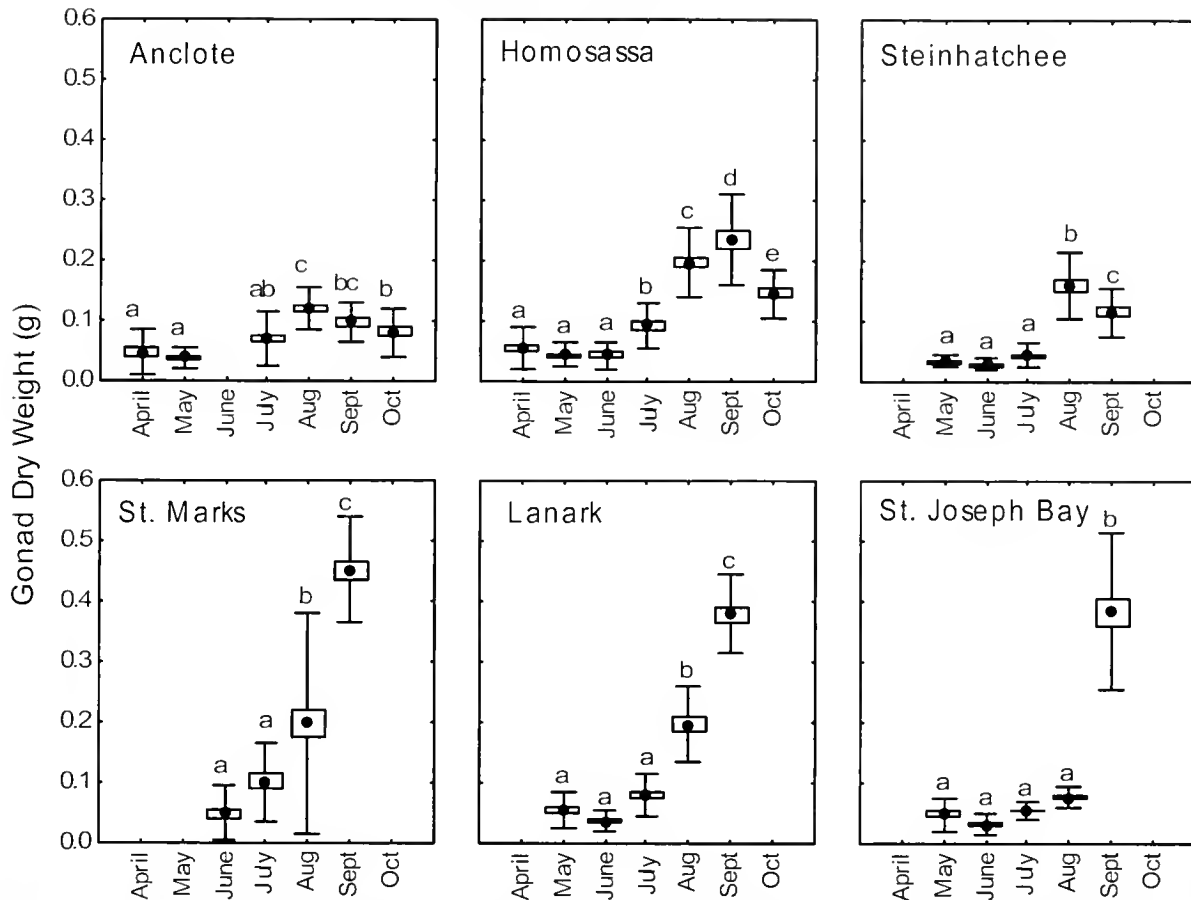


Figure 4. Mean dry weight of gonads of bay scallops, *Argopecten irradians*, from six subpopulations along Florida's Gulf of Mexico coast. Midpoint, mean; box, standard error; bars, standard deviation. Groups that are not significantly different are indicated by the same letter(s).

genesis was occurring without large increases in gonad size during May. Muscle, mantle and digestive tissue weights peaked in August. Further tissue growth was limited to increased gonad size in the early fall, including transfers of stored energy from muscle and viscera to the gonad. Cytoplasmic growth and vitellogenesis were associated with both increases in gonad weight (or body component index) and plateaus then declines in muscle weight (Barber & Blake 1983). The 3-year study found that the initiation of this process varied between the beginning of June and the middle of July but always peaked in September. Their observations compare well with ours.

The variability observed in shell size showed no easily discernable regional pattern. The two sites at which shell sizes were statistically larger than the others at the beginning of the study (Lanark and Anclote) are interspersed spatially among the other four sites. By September, all mean shell heights were around 60 mm at all of the sites. The simplest explanation would be that at or near that size, scallops along Florida's gulf coast begin to use available energy for reproduction. Evidence from recruitment studies supports this claim. At the Anclote and Homosassa sites, recruitment began in August and continued through the fall, whereas very little recruitment was observed in St. Joseph Bay (whose mean shell height was smallest in 2002) until January 2003. Alternatively, St. Joseph Bay animals may have been devoting energy to spawning in the spring of 2002, as suggested by observed recruitment throughout the spring, whereas the Anclote and Homosassa subpopulations did not produce a large number of spring

recruits. The St. Joseph Bay animals may have been in a refractory state during summer of 2002 in preparation for a second major spawning event.

One possible explanation for the discrepancies in sizes between sites is that the subpopulations with smaller mean sizes in May had recruited more recently. The adults used in this study would have recruited sometime during fall 2001 or winter 2002. Evidence from recruitment in fall of 2001 and winter and spring of 2002 does not support the hypothesis for earlier recruitment at Anclote and Homosassa relative to St. Joseph Bay. At the Homosassa site, minor peaks in recruitment occurred in September through October and in December, but some recruits were collected in all months from August 2001 through May 2002. Both the St. Joseph Bay and Anclote subpopulations recruited in fairly distinct peaks during late fall 2001 and early winter 2002, but if anything, the peak recruitment in St. Joseph Bay was earlier, around November 1st, than at Anclote, which peaked closer to December 1st. The overlapping recruitment periods indicate that differences in settlement date were not responsible for the differences in size during May of 2002.

A second possible explanation for the difference in size between subpopulations is that those differences are genetically based. Marelli et al. (1997) and Marelli & Arnold (2001) studied morphological and genetic differences between Florida's Gulf of Mexico bay scallop subpopulations. They suggested that the morphological differences between local subpopulations were minimal and that all of the subpopulations studied were part of the local

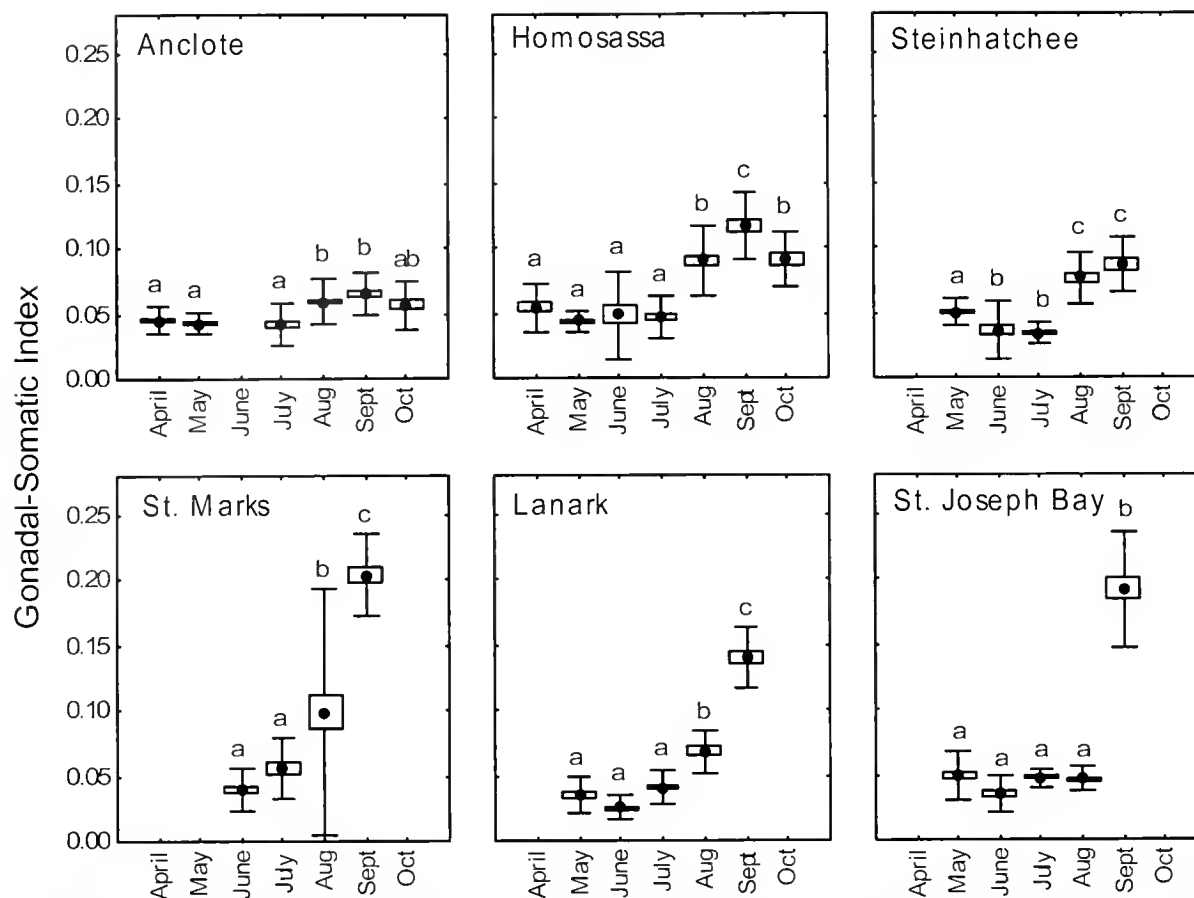


Figure 5. Mean gonadal-somatic indices of bay scallops, *Argopecten irradians*, from six subpopulations along Florida's Gulf of Mexico coast. Midpoint, mean; box, standard error; bars, standard deviation. Groups that are not significantly different are indicated by the same letter(s).

subspecies. In those studies, neighboring subpopulations were often more dissimilar than more distant populations and little or no geographic pattern was apparent within the Florida subpopulations. We conducted size-adjusted principal components analysis on morphological data following Marelli et al. (1997) and found results similar to theirs (unpublished data). There was no readily apparent difference in the shape of any of the scallop subpopulations' shells nor was there a discernable geographic trend (two of the closest subpopulations, St. Marks and Lanark, were the most different). Patterns of gene flow between subpopulations, as influenced by the complex nearshore hydrography of the Florida Shelf (Yang & Weisberg 1999), may affect spatial and temporal patterns of genetic variation. It seems unlikely that any of the differences observed in tissue weights can be explained by genetic variation; rather, small-scale variation in the physical or biotic environments probably resulted in the observed differences in 2002.

Bricelj et al. (1987) summarized the changes in spawning patterns of bay scallops from 3 latitudinally distinct populations: Massachusetts, New York and Florida. The general conclusions were that northern populations showed more discrete peaks in spawning activity, whereas southern populations had more protracted spawning seasons. In the northern populations, periods of somatic growth occurred in spring, prior to spawning, and again in summer and early fall. In the northern populations, a considerable portion of the population will store sufficient energy reserves to overwinter and spawn a second time. These animals will rapidly resume growth of their adductor muscles during the fall. In the current study, we

observed two distinct patterns of growth. In the peninsular subpopulations, changes in muscle and viscera accounted for most of the early-summer growth. Spawning began in late summer, reminiscent of the pattern previously observed in scallops from Massachusetts (Sastry 1970), albeit more protracted in Florida's scallops. Scallops from Florida's Panhandle subpopulations continued to grow until at least the conclusion of our study, and reproduction likely peaked late in the fall, reminiscent of the spawning patterns of populations from the Anclote estuary (Blake & Barber 1983) or North Carolina (Sastry 1970).

The recreational harvest season in Florida begins during a period when scallops are undergoing rapid growth, but the initiation of this period of rapid growth varies between sites. Thus, at the beginning of the season, there is high between-site variability in shell size and adductor muscle mass. The growth is most notable in changes in soft tissues, adductor muscles and gonads. As a result, at some locations, many more scallops could legally be harvested than at others. This finding is especially true if the scallops are shucked at sea. Between July 1st and August 1st, the number of legally harvested shucked scallops declines by about one third because of increases in adductor muscle volume, a process observed at all sites. This decline is less pronounced for whole scallops.

A short delay in the harvest season would result in fewer scallops being taken for harvest, possibly allowing more scallops to survive until fall spawning. A delay in the harvest season would also allow a more simple understanding of harvest, because most



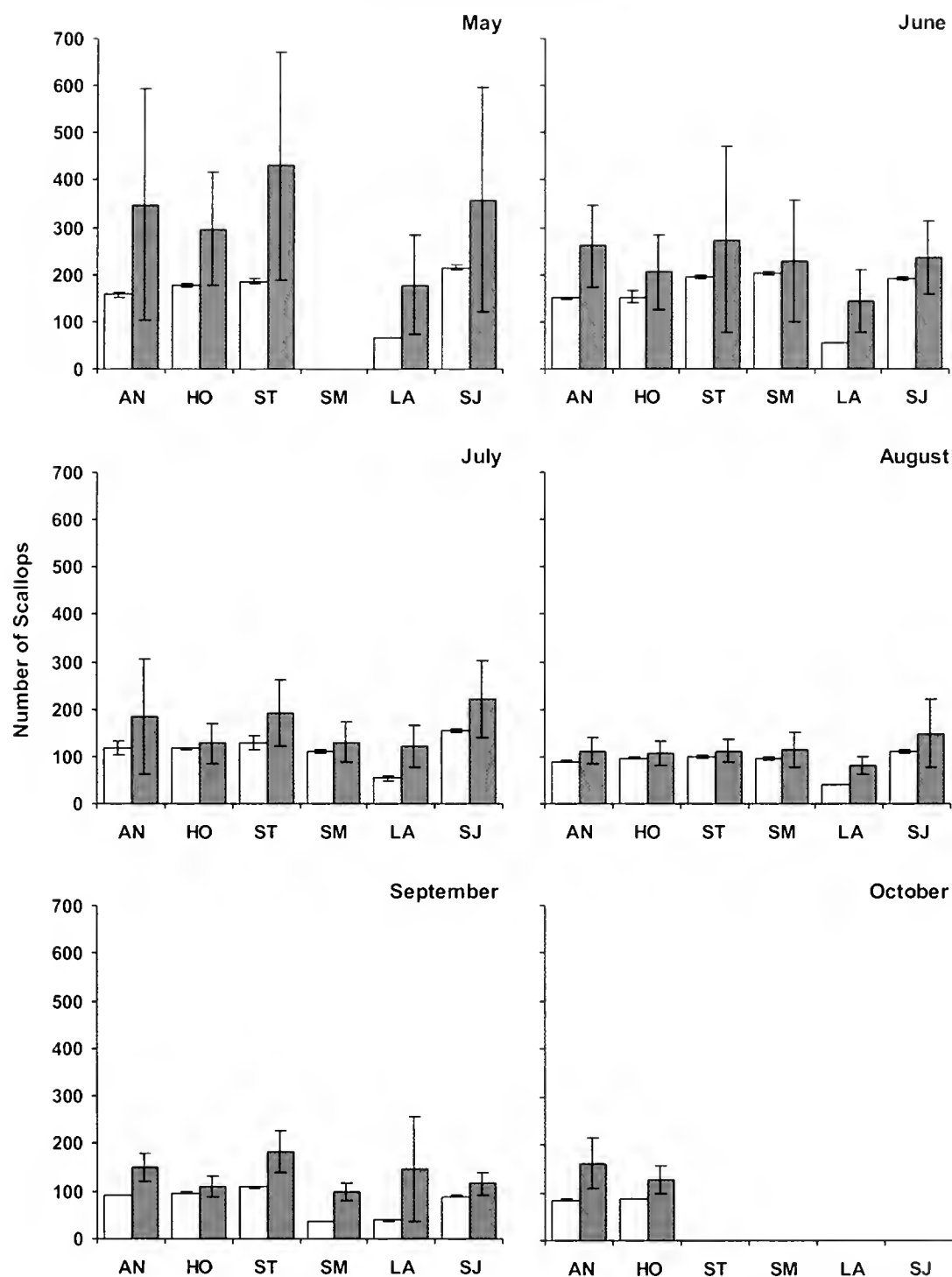


Figure 6. Estimates of recreational harvest limits for bay scallops, *Argopecten irradians*. Open boxes, two gallons whole scallops; shaded bars, 1-pint adductor meats. The collection sites are abbreviated as follows: Anclote, AN; Homosassa, HO; Steinhatchee, ST; St. Marks, SM; Lanark, LA; St. Joseph Bay, SJ.

areas would have a similar number of scallops being harvested by each person collecting their limit. However, natural mortality rates are poorly understood in Florida's scallop subpopulations, and a delayed harvest may not result in a large net gain in the size of the reproductive population. Estimates of instantaneous daily natural mortality in Florida between 0.01 (Greenawalt 2002) and 0.014 (Bologna 1998) indicate that there would be roughly 25% fewer

scallops present on opening day if it were moved from July 1st to August 1st. The effect of delaying the fishing season would then depend on whether fishers continued to collect scallops at the same rate 1 month later (potentially very deleterious) or if fishing mortality, as a percentage of the population, remained steady (little net difference).

Another consideration in changes of the harvest season is that

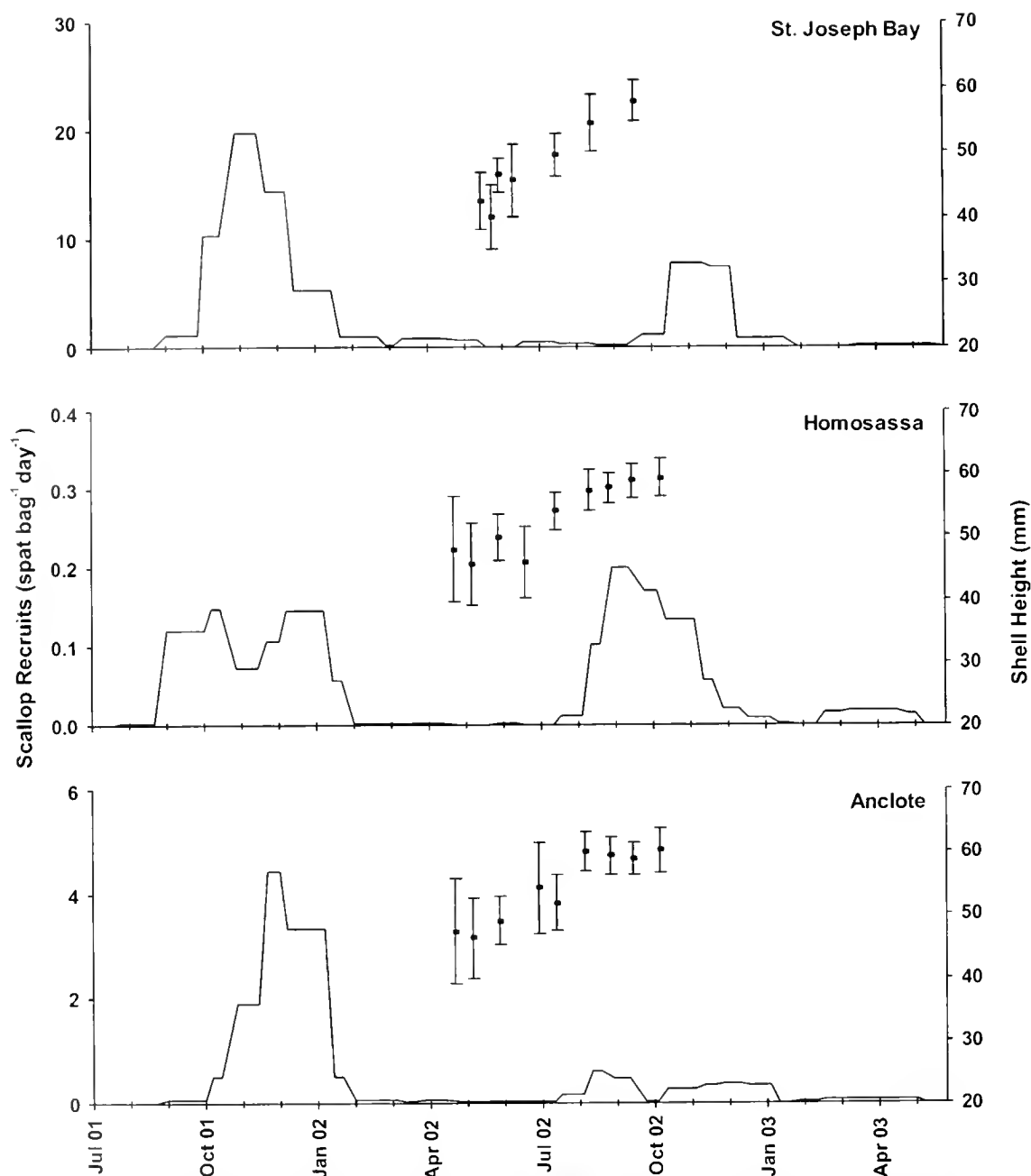


Figure 7. Recruitment rates and adult shell heights (mean  $\pm$  SD) of scallops from three Florida subpopulations of bay scallops, *Argopecten irradians*.

Florida's scallops begin to lose muscle mass in September, as energy is transferred to the gonad (this study; Barber & Blake, 1985). A delay in opening the harvest season, assuming the 10-wk duration was maintained, might actually cause an increased harvest of those scallops that had managed to survive until the fall spawning season. Smaller adductor meats would lead to increased harvest if a person collected the one-pint limit of meat, but the larger shells present in the fall would result in smaller yield for a person collecting a limit of two gallons of whole scallops. A reduction in the duration of the harvest season would probably be unappealing to the tourism industry and recreational fishers unless considerable gains in stability of the population were realized. An analysis of the tradeoffs between biological gain and potential economic

losses resulting from changing the dates of the harvest season should be made before any management actions are proposed.

#### ACKNOWLEDGMENTS

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## REPRODUCTION AND SPAWNING IN CALICO SCALLOPS, *ARGOPECTEN GIBBUS*, FROM BERMUDA

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**ABSTRACT** The gametogenic cycle and breeding season of the calico scallop, *Argopecten gibbus* was examined in Bermuda using quantitative and qualitative methods. Gonadal index varied seasonally, a rapid increase occurring in the winter months from January to March, and reaching maximum values in March and April. Greatest gonad growth and spawning were associated with low seawater temperature and low food levels. Variations in muscle indices implied a partial reliance on muscle reserves during the early stages of oogenesis. The later stages of ovarian growth (vitellogenesis and oocyte maturation) seemed to show a direct dependence on food supply. Histological analysis indicated the initiation of gamete development in early fall, when cell differentiation was first observed; oocyte ripening took place in late fall and early winter, reflected in increased oocyte diameters and gonadal indices. The lack of spawning activity during the hot summer months was seen histologically by the high proportion of spent cells in the gonads and a reduction in oocyte size, resulting in a minimal gonadal index. Individuals with gonad indices above 2 and mean oocyte diameters exceeding 50  $\mu\text{m}$  were induced to spawn with thermal shocks, exceeding 70% response rate in oocyte release in all trials. The present study extends our limited knowledge of the reproductive cycle in this subtropical scallop while providing a useful index to evaluate spawning readiness in cultured broodstock for routine hatchery purposes.

**KEY WORDS:** gametogenesis, scallops, spawning, reproduction, subtropical, *Argopecten gibbus*

### INTRODUCTION

Calico scallops, *Argopecten gibbus*, are largely restricted to the subtemperate and tropical waters of western North Atlantic with the major stocks distributed from Cape Hatteras, North Carolina to the northeastern Gulf of Mexico (Waller 1969); calico scallops have also been collected from the Greater Antilles, Bermuda and the western portions of the Gulf of Mexico (Waller 1969). This species supports a commercial fishery off the East Coast of the United States (Blake & Moyer 1991), and its large-scale culture has been investigated in Bermuda for the purpose of stock enhancement and commercial activity since 1996 (Sarkis et al. 2003). Although much has been reported in the literature for the related bay scallop species, *Argopecten irradians*, little information is available for the calico scallop, especially for those inhabiting Bermuda waters. Data available from the commercial fishery in the United States has generated information on settlement, abundance and yield based on field studies (Roe et al. 1971), population assessments (Cummins 1971) and spawning periods in relation to environmental parameters (Allen 1979, Miller et al. 1981).

Knowledge of the reproductive cycle is necessary for restocking and/or mariculture purposes. Moyer and Blake (1986) have assessed the reproductive cycle of calico scallops in Florida using gonadal indices. However, as documented in *Argopecten irradians*, latitudinal differences occur in the timing of gametogenesis and reproduction; in general, reproductive events occur later in the year and at higher water temperatures in more southern latitudes (Barber & Blake 1991).

The present study describes the gametogenic cycle of *Argopecten gibbus* inhabiting Bermuda waters. Although the use of gonadal indices is a simple, fast and inexpensive method, which may be routinely used in hatcheries, verification of reproductive events pertaining to gamete development is obtained by histological techniques (Couturier & Newkirk 1991). In the present study,

quantitative (gonad index) and qualitative (histology) methods were used to assess the reproductive status of calico scallops. This further allowed for testing the validity of relying on gonadal indices for controlled spawning induction of *A. gibbus* in the hatchery.

### MATERIALS AND METHODS

Calico scallops reared in suspended cultures in Harrington Sound, Bermuda (32°N, 64°W) were collected on a monthly basis from December 2000 to December 2002. Each sample consisting of 30 scallops >50 mm in shell height was transported in water to the pilot hatchery at the Bermuda Biological Station for Research (BBSR) and maintained in an open filtered seawater system for a 24-h period, allowing for gut clearance. Scallops were scrubbed of epibionts and shell height and length measured to the nearest 0.1 mm, with vernier calipers. Scallops were blotted dry and total wet weight was determined using a Sartorius balance (to nearest 0.01 g).

A subsample of 15 scallops was used for determination of gonad and muscle indices on a monthly basis for a two-year period from December 2000 to December 2002; in the second year of sampling, December 2001 to December 2002, a second monthly subsample of 10 scallops was taken simultaneously for histological analyses.

### Tissue Indices

Various condition indices have been used to characterize the apparent "health" of bivalves. Lucas & Beninger (1985) in a critical review of several of these indices, concluded that the dry tissue weight:dry shell weight ratio gave meaningful information about the physiological state of the animal, including reproductive activity. In the present study, gonad and muscle indices were calculated separately; the former reflecting gonad development, and the latter as an indication of seasonal metabolic changes, possibly associated with gametogenesis (Sarkis 1993). Gonads and adductor muscles were dissected, and dried to constant weight in an oven at 80°C. Tissue indices were calculated as follows: (Dry weight of

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tissue/dry empty shell weight)  $\times 100$ ; where a high gonadal index is indicative of mature gonads, and a lower index reflects the onset of gametogenesis or spent gonads.

### Histology

To obtain further insight into oogenesis and oocyte maturation, samples of gonads were dissected monthly and prepared for histological analysis. A small distal portion of the gonad (5 mm  $\times$  5 mm) was fixed in Bouin's fluid for 48–72 h and stored in 70% ethanol until sectioning (Naidu 1970). Fixed ovarian tissue samples were processed for histology according to standard methods for marine bivalves (Howard et al. 2004). The fixed tissues were dehydrated through an ascending alcohol series, embedded in wax, cut to 5- $\mu$ m sections and stained with hematoxylin and eosin.

Thin sections were examined under a compound microscope ( $\times 100$  magnification) for developmental purposes and categorized into 6 stages based on Naidu (1970) and Couturier and Newkirk (1991) as follows—Stage 1: undifferentiated, follicular structure is indistinct, and no sex cell primordia; Stage 2: differentiated, oocyte development commences, primary oogonia are visible, sex cells range from 15–25  $\mu$ m in diameter; Stage 3: developing, mostly previtellogenic and vitellogenic oocytes, follicle lumen is open or only partially occluded, and oocyte diameter ranges from 30–70  $\mu$ m; Stage 4: ripe, gonads show packed follicles, the follicular lumen is occluded, there is no interfollicular space visible, and oocytes range 60–70  $\mu$ m in diameter; Stage 5: spawning, the follicle lumen is opening, follicle size diminishing and ripe oocytes range 60–70  $\mu$ m; Stage 6: spent, follicles are empty and frequently filled with hemocyte aggregations on slide preparations, and an occasional atretic oocyte is present.

Each scallop was categorized according to one of the previous gonad development stages, and the frequency (%) of each stage determined for monthly samples. The data were plotted as cumulative histograms while assessing the developmental stage of each scallop under the microscope, the diameters of 10 oocytes were measured randomly in each individual using an ocular micrometer. Photomicrographs of representative developmental stages were obtained under  $\times 100$  magnification using a digital photomicroscopy setup (see Fig. 3 later).

### Spawning Induction

Results from spawning induction, performed during the course of four years of hatchery operation (1996–1998, 2002), are presented here for comparison with conclusions drawn from in-depth histological data determined within the scope of this study. Cultured adults were obtained from field sites during winter/early spring (December to April), and the gonadal index calculated as previously described on a subsample. Spawning induction was attempted on 30 scallops by standard thermal cycling shocks (Sarkis et al. 2003, Bourne et al. 1989). The proportion of spawning females was calculated for each trial, as an indication of spawning success. Comparisons were made between the percentage response and histological state.

## RESULTS

### Tissue Indices

Gonadal and muscle indices for the calico scallop reared in Bermuda waters are shown in Figures 1 and 2, respectively. Al-

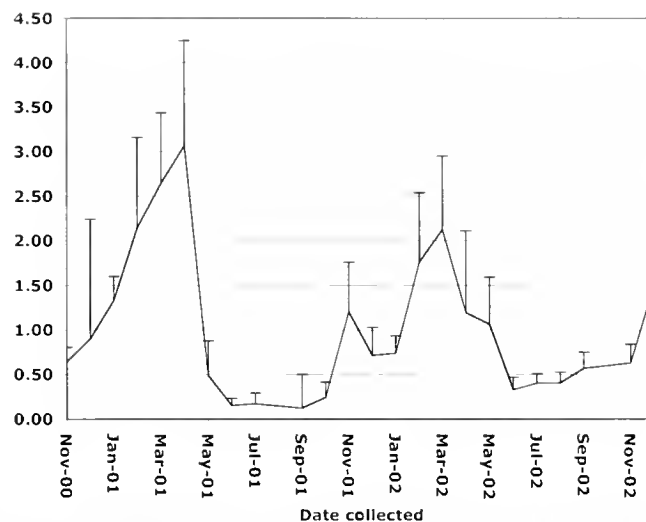


Figure 1. Seasonal gonadal index of cultured *Argopecten gibbus*. Data points represent mean  $\pm$  SD ( $n = 15$ ).

though fluctuations in gonadal indices were large, especially in the first year of sampling, the trend was for maximum values of the index during the winter months, and minimum values during the summer months. There was a slight increase in gonadal indices during the autumn months (September to December), remaining relatively low until December (0.2–0.9). Thereafter, a rapid increase in gonad weight occurred in the winter months, from January to March, reaching maximal values in March and April.

The time required for scallops to attain maximum gonad index varied slightly from year to year, with maximum values observed in April in 2001 ( $3.07 \pm 1.18$ ), and in March during 2002 ( $2.13 \pm 0.83$ ). Variation in gonad condition among individual scallops was large particularly throughout the spawning season from February to May, implying perhaps low synchronicity in spawning among conspecifics. Gonadal indices were at their lowest during the summer months from June to August, ranging from 0.12 ( $\pm 0.38$ ) to 0.17 ( $\pm 0.12$ ) in 2001, and 0.33 ( $\pm 0.14$ ) to 0.41 ( $\pm 0.12$ ) in 2002, representing a "rest" period.

The trend in adductor muscle indices was not as distinct as that seen for gonadal indices and seasonal fluctuations were not as wide, although they were apparent (Fig. 2). Maximum values were generally recorded at the end of the summer months in September,

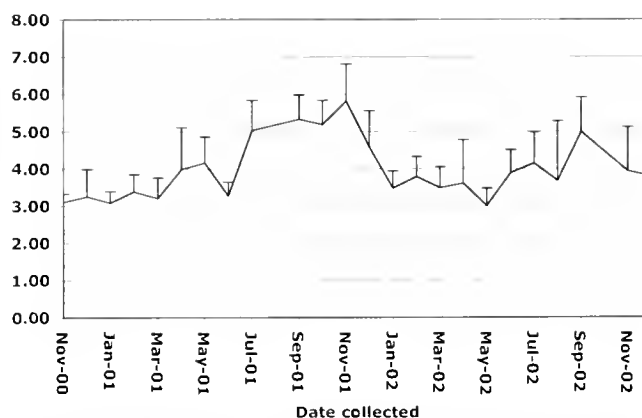


Figure 2. Seasonal variation in adductor muscle index in cultured *Argopecten gibbus*. Data points represent the mean  $\pm$  SD ( $n = 15$ ).

reaching  $5.33 \pm 0.65$  in 2001 and  $4.99 \pm 0.92$  in 2002. These coincided with low gonadal indices (compare Figs. 1 and 2). Muscle indices decreased throughout the autumn, as gonadal indices started to increase. As gonadal indices peaked from January to April, muscle indices remained low, fluctuating between  $3.01 \pm 0.47$  and  $4.06 \pm 1.03$  in both years of the study. Variations among individuals were seen on a monthly basis, as for gonadal indices.

In summary, gonadal indices indicated large seasonal fluctuations with the occurrence of well-defined peaks of gonadal activity during the winter months (January to April), and a period of inactivity during the summer months. Muscle indices showed, to a certain extent, inverse seasonal fluctuations to those recorded for gonadal indices.

#### Histology

Photomicrographs of ovarian developmental stages are shown in Figure 3. Each stage is easily identified by the type of cells

present or absent, the shape of the follicles containing gametes, and the size of the oocytes.

The first appearance of cell differentiation was observed in September, when the gonad consisted of 60% developing cells (Fig. 4). The first appearance of ripe oocytes was recorded in December, and the gonad at this time consisted mainly of developing and ripe cells (90% in 2001 and 100% in 2002) (Fig. 4). Oocyte ripening occurred during the winter months, from December to March, as the proportion of ripe cells increased to 80%, 90% and 70% of the gonad in February, March and April, respectively (Fig. 4). During these three months, the gonad samples consisted of 100% ripe and spawning individuals, implying a readiness to spawn. Between April and June, the proportion of spawning and spent individuals increased, indicating the end of spawning activity (Fig. 4). By the month of August, 100% of the scallops seen were spent and contained no gametes (Fig. 4).

Seasonal variations in mean oocyte diameter are recorded in

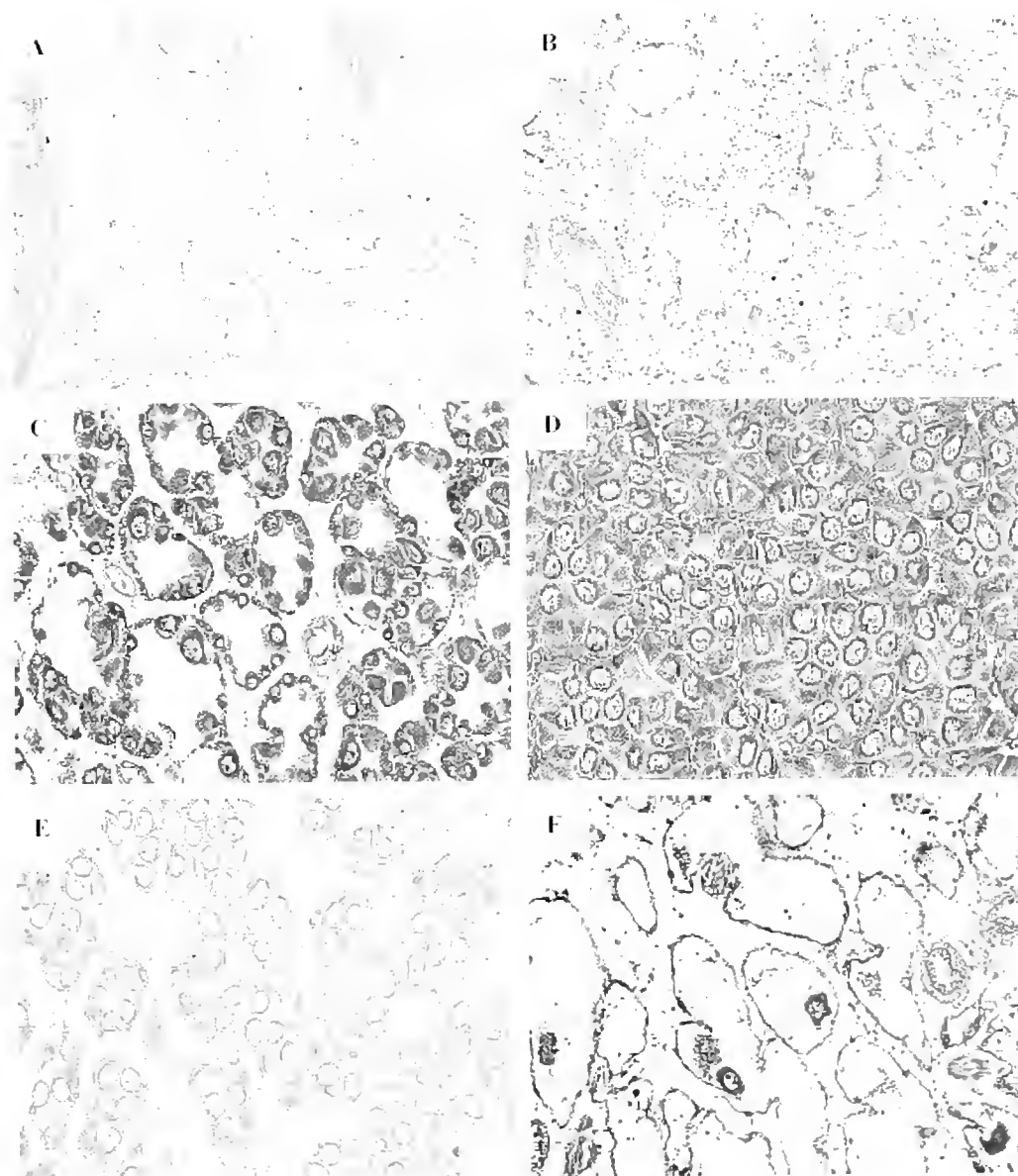


Figure 3. Stages of ovarian development in cultured *Argopecten gibbus* ( $\times 100$  magnification). A, undifferentiated; B, differentiated; C, developing; D, ripe; E, spawning; F, spent.

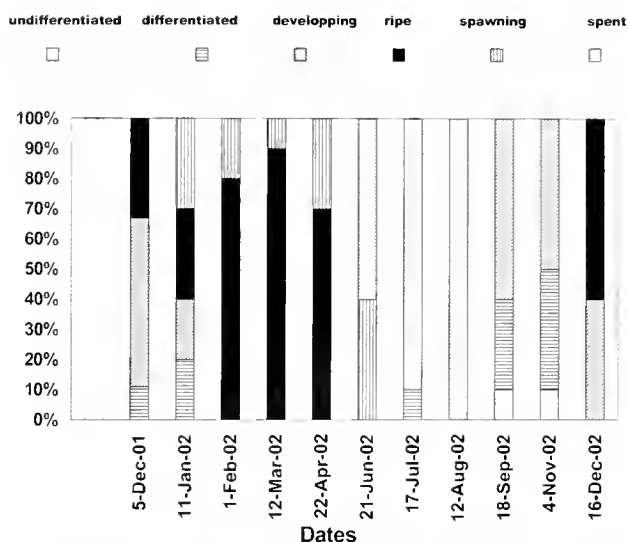


Figure 4. Qualitative assessment of reproductive patterns in the ovarian portion of the gonad of cultured *Argopecten gibbus* from Bermuda.  $n = 10$  animals per sample date.

Figure 5. Microscopic observation of histological sections of ovaries revealed that oocyte size was comparable in individuals collected during oocyte ripening in the winter, from December to April, ranging from 53.7  $\mu\text{m}$  to 63.5  $\mu\text{m}$ . The dramatic decrease in oocyte size to 23.1  $\mu\text{m}$  seen from April to June coincided with the histological evidence of spawning and the lack of gametogenic activity seen during the hot summer months.

#### Spawning Induction

Results obtained in the hatchery from spawning induction trials yielded a spawning success of 70% to 90% of the females induced (Fig. 6) when gonadal indices exceeded 2. Such individuals generally responded to the thermal treatment and released gametes within 90 min of stimulation and most individuals did so in a synchronous manner. Attempts to spawn individuals with gonadal indices below or equal to 2 resulted in variable success with generally fewer than 40% of individuals responding to the thermal shock (Fig. 6).

#### DISCUSSION

The present study suggests that calico scallops in Bermuda undergo reproduction mainly from December to April, and possi-

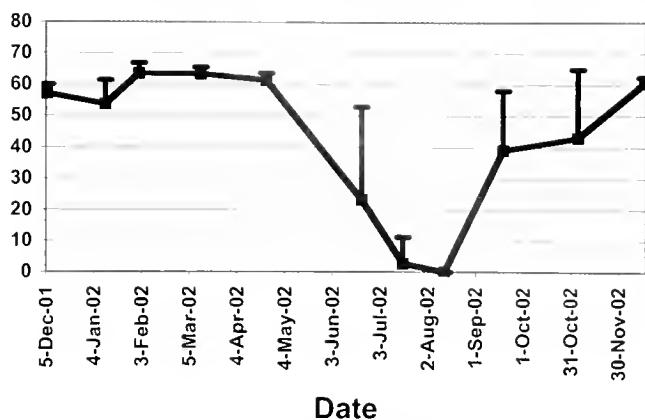


Figure 5. Seasonal variation in mean oocyte diameter in culture *Argopecten gibbus*. Points represent the mean + S.D. ( $n = 100$ ).

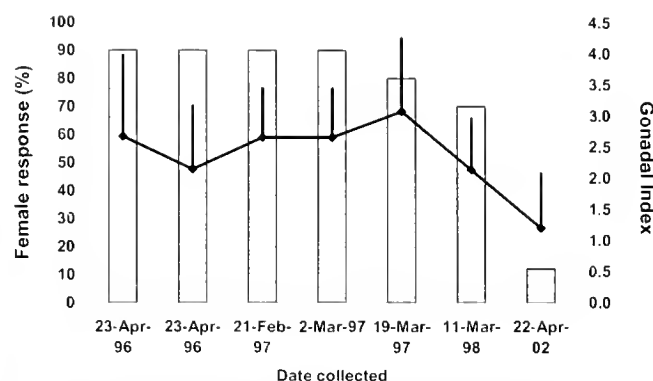


Figure 6. Relationship between gonadal indices (line, mean + SD,  $n = 15$ ) and female spawning response (bars, %,  $n = 30$  on each attempt) following thermal stimulation of calico scallops in the hatchery.

bly May. Gamete differentiation and development began in September, associated with a mean oocyte size of  $<20 \mu\text{m}$ , low gonadal indices and maximal muscle indices. Oocyte development occurred throughout the fall, coinciding with a gradual increase in mean oocyte diameter and gonad weight, and a reduction in adductor muscle indices. The first ripe scallops appeared in December when mean oocyte diameter exceeded  $50 \mu\text{m}$  and gonadal index approached 1. Oocyte ripening continued during the winter months, associated with low muscle indices and a rapid increase in gonad weight yielding by February a gonadal index close to or above 2 with 80% of the scallops classified as ripe and showing some evidence of spawning in histological sections. The spawning period extended into March and April, and was reflected in the responses obtained from controlled spawning trials at the BBSR hatchery, where induced individuals released gametes between February and April. Optimal spawning responses were obtained when gonadal index was above 2, coinciding with histological analyses showing ovaries with high percentage of ripe oocytes ( $>70\%$ ). After the spawning period, both gonadal index and oocyte diameters declined rapidly in late spring/early summer, and histology showed increased proportions of spent individuals. By August, gonads consisted of spent cells only, resulting in minimal gonadal indices and coinciding with a lack of spawning activity during the summer months.

The spawning period of the calico scallop in Bermuda, extending over the winter months, is associated with colder water temperatures and lower food availability. This cycle differs from that recorded for the same species in Florida (Moyer & Blake 1986), where two spawning periods are observed, the first in late spring (April to June), and the second in the autumn. As mentioned previously, latitudinal differences in gametogenesis have been recorded within a species, explained in part by spatial differences in environmental influences, namely those of food and temperature (Barber & Blake 1985, MacDonald & Thompson 1988).

Temperature is most often cited as influencing bivalve reproduction and triggering spawning. Gamete maturation for *Argopecten gibbus* in Bermuda coincided with minimal sea surface temperatures ranging from  $16^{\circ}\text{C}$  to  $18^{\circ}\text{C}$  (Doug Connelly, unpublished data). Blake & Moyer (1991) found that the required threshold temperature for gonad development and ripening for the same species in Florida was of  $19^{\circ}\text{C}$  to  $20^{\circ}\text{C}$ . They further concluded that temperatures above  $22^{\circ}\text{C}$  stopped gonad ripening and spawning did not occur, which agrees with the results in the present study where spawning terminated in late spring at temperatures above



21°C, and a lack of gametogenic activity was seen during the warmer months (July and August), when sea surface temperatures reached a maximum (29°C) (Doug Connelly, unpublished). However, in keeping with the annual variations in gonadal indices reported in the present study, Manuel (2001) recorded yearly fluctuations in peak spat settlement periods in the field. These variations may be associated with changes in sea surface temperatures and food supply between years, because environmental characteristics of Bermuda's inshore waters, habitat to these scallop species, have been known to be wind-driven and hence highly variable; this is especially apparent in the timing of "bloom" conditions, expressed as chlorophyll *a* (1.93 µg chl *a* L<sup>-1</sup>), occurring during late fall or early winter (November and December) (Doug Connelly, unpublished). The trend seen in the muscle index points to the relationship between "bloom" occurrences and gonadal development in the early stages of oogenesis, and it implies to a certain extent a direct dependence on external food supply for oocyte ripening during periods of low food availability in late winter/early spring (0.42 µg chl *a* L<sup>-1</sup>) (Doug Connelly, unpublished). Similar conclusions were drawn by Manuel (2001) for another scallop species inhabiting Bermuda waters, *Euvola (Pecten) ziczac*.

Thermostimulation has proven efficient and rapid for inducing spawning in calico scallops in Bermuda with a mean gonadal index above 2, compared with other species (Monsalvo-Spencer et al. 1997). To date, thermal shock seems to be the most efficient method, for inducing sperm and ova release in hatchery scallops (Monsalvo-Spencer et al. 1997), and this is especially true for pectinidae, where a critical temperature range or minimum threshold temperature has been most frequently implicated in the initiation of spawning (Barber & Blake 1983, Moyer & Blake 1986).

However, variable responses have been obtained using thermal shock induction in Bermuda with scallops having a gonadal index close to or below 2. This points to the influence of other factors in controlled spawning events, perhaps related to hatchery procedures and/or to natural spawning cues. For example, it has been shown that spawning in cultured and natural populations of various scallops does not appear to occur until the majority of the animals in a population have ripe gonads, normally above 70% or more of the populations (Lubet et al. 1991, Couturier 1994, Lohrmann & Von Brand 2005). In this study, fluctuations seen in gonadal index among individuals on a monthly basis indicate a heterogeneity within the sample, and should be given closer consideration when mean index is low; it may well be that sample size for hatchery spawning induction needs to be increased at this time, to ensure a larger proportion of ripe animals. In addition, it may be worthwhile investigating the influence of other spawning cues on calico scallops in Bermuda. Several of these, such as salinity, lunar phase, light, dissolved oxygen, pH, mechanical shock and various chemicals and food pulses have been examined for other pectinid species through correlation with field observations and more rigorous laboratory experiments (Gibbons & Castagna 1984, Starr et al. 1990, Velez et al. 1990, Desrosiers & Dubé 1993, Jamaillo et al. 1993, Couturier 1994).

In conclusion, it seems necessary for the calico scallop to have a mean gonadal index value >2 for a good response to spawning induction via thermal shocks. It can be further added that the use of gonadal indices for routine hatchery purposes provides a good snapshot of the reproductive status. However, to have a thorough understanding of gametogenic events in a specific population in relation to gonadal indices, histological assessments of reproduction in scallops is warranted in a first instance.

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## ASSORTATIVE FERTILIZATION IN *CHLAMYS FARRERI* AND *PATINOPECTEN YESSOENSIS* AND ITS IMPLICATION IN SCALLOP HYBRIDIZATION

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**ABSTRACT** Assortative mating and reduced hybrid fitness are typically invoked to explain the stability of hybrid zones and the specification of two closely related taxa. Such examples of mate preferences in nature, especially for broadcast free-spawning marine invertebrates, and the understanding of the underlying mechanisms responsible for this positive selection are still lacking. This study documents another example of assortative fertilization by genome *in situ* hybridization in reciprocal crosses of scallops *Chlamys farreri* and *Patinopecten yessoensis*. Females produced eggs selected sperm nonrandomly but showed significant gamete preference of conspecifics over heterospecifics, with *Chlamys farreri* females using average 89.1% of conspecific sperms and the *Patinopecten yessoensis* using average 94.3% in natural spawning conditions (temperature 17°C, salinity 31.5‰). This conspecific gamete advantage may be related to the coordinated communication between interacting gametes, and the conspecific binding genotype selection during the communicational process may also be involved. Two environmental factors, temperature and salinity were also tested to determine to what extent they affect the patterns of assortative fertilization between these two species. The result showed that temperature seemed to have more influence on assortative fertilization than salinity did. At the temperatures of 11°C, 17°C, 22°C and 26°C, the average conspecific sperms preference of *Chlamys farreri* eggs was 80.0%, 89.1%, 95.1% and 95.5% respectively, whereas at the salinity of 24‰, 28‰, 31.5‰ and 35.5‰, the average mating preference was 97.2%, 91.2%, 89.1% and 99.5% respectively. These results may have some bearings on the condition dependent alteration in the inter gamete recognition system. Alternatively, it may be simply the byproduct of different sperm tolerance ability under different conditions. The implication it may provide to hybrid production was also discussed.

**KEY WORDS:** scallop, assortative fertilization, *Chlamys farreri*, *Patinopecten yessoensis*

### INTRODUCTION

Many theories have been developed to interpret the formation of reproductive isolation and hence the speciation process between two sympatric closely related taxa in recently years. (Palumbi & Metz 1991, Metz et al. 1994, McCartney et al. 2000, Knowlton 1993, Coyne 1992) These theories generally fall into two categories: pre- and post-zygotic barriers. Pre-zygotic barriers include mechanisms such as mate choice, genital incompatibility, spatial and temporal habitat differences and gamete incompatibility. Post-zygotic barriers, on the other hand, include mechanisms such as embryo inviability and hybrid sterility (Dobzhansky et al. 1977, Templeton 1989, Lessios 1998). Whereas, assortative fertilization or gamete preference, which is commonly regarded as a post-mating, pre-zygotic isolating mechanism, has only recently come to prominence and is increasingly accepted as another important possible barrier to hybridization and gene flow between closely related species (Bierne et al. 2002, Geyer & Palumbi 2005).

Assortative fertilization is a subtype of fertilization barrier that depends upon characteristics of the female and male such that gametes from like or unlike parents have a greater or lesser than random chance of uniting (Markow 1997). Evidence of such kind of gamete preference has been recorded in many terrestrial species such as *Drosophila* (Chang 2004, Markow 1997), crickets (Howard & Gregory 1993, Howard et al. 2002) and flour beetles (Robinson et al. 1994, Wade et al. 1995). These studies indicated without exception that conspecific sperm precedence, or biased use of sperm from males of one species might occur when a female was

exposed to sperm from males of multiple species. Possible mechanism underlying was also highly varied. In some cases, it involves compatibility of genitalia or gamete morphology. For example, in *Rhododendron*, overgrowth or undergrowth of heterospecific pollen tubes yields few hybrid embryos (Williams & Rouse 1990). In other cases, it seems to be related to functional or physiological interactions between sperms and the female reproductive tract (Markow 1997). In ladybird beetles heterospecific sperm are inactivated in storage in female reproductive tract (Katakura 1986, 1997). In *Drosophila* conspecific sperm just incapacitates or displaces heterospecific sperm during the storage. But for the broadcast free-spawning marine invertebrate, in which courtship and other complex mating behaviors that facilitate mate choice are generally non-existent, the opportunity for conspecific sperm preference and the possible mechanism has been largely ignored. To date, only few marine invertebrates have been studied for such gamete preference (Bierne et al. 2002, Palumbi 1999, Geyer & Palumbi 2005, Yung & McCartney 1994), and the possible mechanism seemed even more complicated than that of terrestrial species because of the smaller arena, the surface of gametes and the seawater between them, in which the assortative fertilization could take place. Here we present another example of assortative fertilization in external fertilization species of marine bivalves.

Hybridization between scallops *Chlamys farreri* and *Patinopecten yessoensis*, both of which are economically important shellfish species along the coast of China and Japan, has been intensively studied in recent years with the aim of improving *Chlamys farreri* germ quality from high rate of mortality and disease susceptibility (Yang et al. 2004, Zhou et al. 2005). No apparent gametic incompatibility was found in crosses of both directions between these two species either in small-scale laboratory

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experiment or in larger scale breeding program (Yang et al. 2004). The putative hybrid was believed to be able to live successfully through planktonic larval stages and grow into full adults with heterosis in growth and disease resistance (Yang et al. 2004), but in some cases of hybridization, contamination was detected by genetic examination of the progeny despite much care being taken in the hybridization process (Lü et al. in press). The same phenomenon was found in the hybridization of many oyster species of *Crassostrea* (Allen & Gaffney 1993, Allen et al. 1993). Sometimes the contamination rate is comparatively high given the fact that the concentration of contaminated sperm (if exists) was much lower than that of heterospecific sperm added in hybridization. We suspect assortative fertilization may be responsible for this problem. So the aim of this study was to investigate (1) if there also exists assortative mating phenomenon between species of scallops as found in other marine invertebrates; (2) to what degree the presence of conspecific sperms affect the cross-fertilization success of interspecific sperm and (3) to what extent this assortative fertilization can be affected by environmental factors, such as temperature and salinity, so that measures may be taken to improve the scallop hybridization program.

## MATERIALS AND METHODS

### Scallops and Gametes

*Chlamys farreri* were collected from the Jiaonan area of Qingdao and *Patinopecten yessoensis* were obtained from the Dalian coast of Bohai Sea in early April 2005. Both species were transported to the laboratory and temperature and feeding were adjusted to synchronize maturation among species. All gametes were obtained by induced spawning with drying and thermal shock. Oocytes from individual females were gently sieved and placed into separate beakers after the quality was checked by light microscope and no fertilization was confirmed, counted using a Coulter cell counter and adjusted to  $1 \times 10^6$  per female. Sperms from individual *C. farreri* and *P. yessoensis* were placed into separate beakers. Same treatment was conducted and adjusted to  $1 \times 10^8$  per male.

### Experimental Design

Four gamete mixtures were produced in the experiment: A mix of oocytes from 20 female *C. farreri* with a finally total number of  $2 \times 10^7$ , a mix of oocytes from 20 female *P. yessoensis* with a finally total number of  $2 \times 10^7$ , a mix of spermatozoa from 20 male *C. farreri* with a finally total number of  $8 \times 10^8$  and a mix of spermatozoa from 20 male *P. yessoensis* with a finally total number of  $8 \times 10^8$ . All the mixtures were then further divided into equal amount of portions, each used for one cross. This guarantees almost equal amount of gametes used in each cross and obtain a 40:1 sperm: egg ratio for the fertilization. The six crosses were conducted at temperature 17°C and salinity 31.5‰ (natural spawning condition); *C. farreri* (♀) × *C. farreri* (♂); *C. farreri* (♀) × *P. yessoensis* (♂); *P. yessoensis* (♀) × *P. yessoensis* (♂); *P. yessoensis* (♀) × *C. farreri* (♂); *C. farreri* (♀) × [*P. yessoensis* (♂) + *C. farreri* (♂)]; *P. yessoensis* (♀) × [*P. yessoensis* (♂) + *C. farreri* (♂)]. The first four crosses were used as positive controls and the later two allowed for interspecies sperm competition with equal amount of sperm from both species added. Another six sperm competition crosses were conducted the same way with the *C. farreri* (♀) × [*P. yessoensis* (♂) + *C. farreri* (♂)] under 3 levels of

temperature (11°C, 22°C and 26°C) at the salinity 31.5‰ and 3 levels of salinity (20‰, 28‰ and 35.5‰) at the temperature 17°C. The temperature was maintained by using a bio-chemical incubator (L15-2, SHELLAB) and the salinity was maintained by just adding fresh water or crude salt. All the crosses were performed in four replicates and conducted simultaneously.

### Larval Development and Genetic Confirmation

Fertilization was allowed to proceed for a minimum of 50 min in each cross before all the eggs were transferred to a culture tank with a density of 30 eggs/mL. Normal culturing procedure was followed afterwards. Fertilization success was determined by examining at least 200 oocytes by light microscope at 60–90 min post-insemination. Two replicate subsamples were collected from each duplicate of the crosses for each sampling. The larvae were collected after they reached early trochophore stage and the hatching rate was also determined.

The metaphase chromosome was prepared following the procedure described by Wang et al. (1990). In brief, the embryos were exposed to 25% sea water for about 50 min after 40 min of culture in 0.04% colchicines at room temperature, then they were fixed with Carnoy fixatives (ethanol: glacial acetic acid = 3:1), and stored at -20°C until use. The fixed embryos were dissociated into fine pieces by pipetting in 50% acetic acid in a 1.5 mL microcentrifuge tube. The resultant cell suspension was dropped onto hot-dry glass slides and air-dried. Chromosome preparations were preserved in a moist chamber until use.

The GISH (genome *in situ* hybridization) procedure followed the protocol described by Leitch et al. (1994) and Takahashi et al. (1997) with some modifications. Briefly, chromosome slides were denatured in a mixture containing 75% formamide and 2 × SSC for 2–3 min at 72°C, dehydrated through an ice-cold ethanol gradient (70%, 90% and 100%), 5 min each, and air-dried. Genomic probe mixture was denatured for 5 min at 80°C, followed by immediately being put on ice for at least 10 min. The probe hybridization mix was applied to the slides and *in situ* hybridization was carried out at 37°C for 14–18 hr. The slides were washed twice in 2 × SSC, and 50% formamide at 42°C for 10 min, 1 × SSC at 42°C for 10 min and finally in 2 × SSC at room temperature for 10 min after the hybridization. Biotinylated probes were detected with fluorescein isothiocyanate conjugated avidin DCS (FITC) (VECTOR) for 1h at 37°C, counterstained with Vectashield mounting medium with propidium iodide (PI) (VECTOR) for 40 min at 37°C. Hybridization signals were detected by using a Nikon E-800 microscope equipped with the appropriate filter sets for FITC and PI.

### Statistical Analysis

The percentage of hybrid larvae was determined by counting the hybrid rate in at least 200 metaphase chromosomes displayed by GISH. Two replicate subsamples were collected from each duplicate of the crosses for each sampling. These percentages were compared with random expectation by a  $\chi^2$  test of the observed number of hybrid offspring versus the number expected, if sperm use was random. The expectations were based on the measured concentrations of sperm used in each cross and the average larval hatching rate of each control. It was calculated as  $EP = S1 \times H1 / (S1 + S2) \times H2$ , where S1 and S2 are the respective sperm concentrations, and H1 and H2 are the respective average larvae hatching rates. Hybrid probabilities were calculated according to Palumbi (1999) as  $SR / (SR + 1)$ , where  $SR = (L1/L2) \times (S2/S1)$ , L1

TABLE 1.

The mean fertilization rates and the larvae hatching rates ( $\pm$ SE) in six crosses in natural spawning conditions (temperature 17°C and salinity 31.5‰), C: *Chlamys farreri* and P: *Patinopecten yessoensis* ( $n = 8$ )

Crosses	Fertilization Rate	Larvae Hatching Rate
C (♀) × C (♂)	96.5 ± 2.6	91.2 ± 1.9
C (♀) × P (♂)	95.3 ± 2.3	90.0 ± 1.9
P (♀) × P (♂)	98.0 ± 1.7	92.0 ± 1.1
P (♀) × C (♂)	96.2 ± 2.0	89.5 ± 2.2
C (♀) × [P (♂) + C (♂)]	97.4 ± 3.4	90.6 ± 2.5
P (♀) × [P (♂) + C (♂)]	94.0 ± 3.1	87.0 ± 1.6

and L2 are the number of larvae sired by heterospecific and conspecific males and S1 and S2 are the respective sperm concentrations. The percentage of hybrid larvae from each sperm competitive cross in changed conditions was also compared by a  $\chi^2$  test with that of in natural spawning conditions.

## RESULTS

### Fertilization and Genetic Confirmation in Control Crosses

In this part, four independent crosses with replicates were performed as positive control. Almost no difference in fertilization and the larval hatching rates was found among the crosses of *C. farreri* (♀) × *C. farreri* (♂); *C. farreri* (♀) × *P. yessoensis* (♂); *P. yessoensis* (♀) × *P. yessoensis* (♂) and *P. yessoensis* (♀) × *C. farreri* (♂). All crosses showed high levels of fertilization rates and larval hatching rates (Table 1). This is in agreement with what found by Yang in 2004. Genetic confirmation by GISH showed that all offspring from crosses of *C. farreri* (♀) × *P. yessoensis* (♂) and *P. yessoensis* (♀) × *C. farreri* (♂) were hybrid (Fig. 1b). No evident gamete incompatibility was found in these two species.

### Fertilization and Genetic Confirmation In Sperm Competitive Crosses

High fertilization and the larval hatching rates were also found in the two crosses of *C. farreri* (♀) × [*P. yessoensis* (♂) + *C. farreri* (♂)] and *P. yessoensis* (♀) × [*P. yessoensis* (♂) + *C. farreri* (♂)] at control condition (Table 1). But genetic confirmation by GISH showed that the hybrid rates of the offspring from these crosses departed significantly from equal species contribu-

tion ( $P < 0.005$ ) when sperm choice was available. The average hybrid rates were found to be 10.9% in *farreri* (♀) × [*P. yessoensis* (♂) + *C. farreri* (♂)] and 5.7% in *P. yessoensis* (♀) × [*P. yessoensis* (♂) + *C. farreri* (♂)] (Table 2, Fig. 2). The expected hybrid rates based on the measured concentrations of sperm used in each cross and the mean larval hatching rate of each control were 49.3% and 48.6% respectively if the sperm use was random. The hybridization probability was 0.112 and 0.055 respectively. Females of both *C. farreri* and *P. yessoensis* showed strong biased sperm use when their oocytes were exposed to mixed sperms from the two species.

### Environmental Effect of Assortative Fertilization

The fertilization and larval hatching rate in crosses of *C. farreri* (♀) × [*P. yessoensis* (♂) + *C. farreri* (♂)] decreased radically when the temperature or the salinity came to extreme with a less than 60% fertilization rate and hatching rate was found at salinity 31.5‰ (the data not shown). The hybrid rate in crosses of *C. farreri* (♀) × [*P. yessoensis* (♂) + *C. farreri* (♂)] decreased significantly with increasing temperature (Table 3, Fig. 2). At the temperatures of 11°C, 22°C and 26°C, the mean hybrid rates were 20.0%, 4.9% and 4.5% respectively. These departed significantly from the mean hybrid rate obtained in natural spawning condition ( $P < 0.05$ ). The salinity also showed significant impact on the assortative fertilization in the same manner with the mean hybrid rates of 3.8%, 8.8% and 0.5% at the salinity of 24‰, 28‰ and 35.5‰ (Table 3, Fig. 2). Temperature seemed to have more influence on assortative fertilization than salinity did.

## DISCUSSION

Assortative fertilization is believed to be a very important mechanism in explaining barriers to gene flow and hybridization between many sympatric closely related species (Bierne et al. 2002). However, it is not our interest in this study to investigate whether it is also important in the speciation or the reproductive isolation between *Chlamys farreri* and *Patinopecten yessoensis*. Because the asynchronous spawning, which alone would suffice to prevent any form of hybridization in nature, has been intensively developed between these two species. The peak spawning time of *C. farreri* along north coast of China was from early May to mid June at water temperature of 16° to 22°C (Wang et al. 1993), whereas *P. yessoensis* spawned in a colder water temperature of 8°C to 8.5°C commonly from late March to mid April (Wang et al.

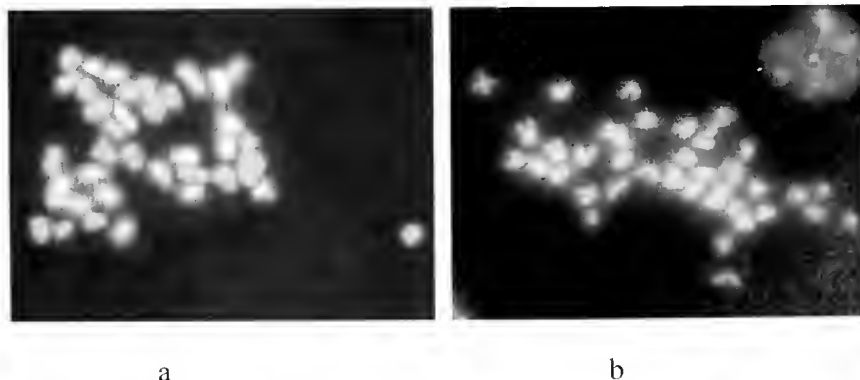


Figure 1. The representative metaphase chromosomes of hybrid and pure species larvae confirmed by GISH at trochophore stage: a, shows metaphase chromosomes of hybrid larvae and b, shows metaphase chromosomes of pure species larvae of *Chlamys farreri* by biotinylated probes of *Chlamys farreri*

TABLE 2.

The mean hybrid rate ( $\pm$ SE) of sperm competition crosses in natural spawning conditions (temperature 17 °C and salinity 31.5‰). The percentage was compared with random expectation by a  $\chi^2$  test of the observed number of hybrid offspring versus the number expected if sperm use was random. Hybridization probability was calculated as  $SR/(SR + 1)$ , SR is the average proportion of hybrids observed, corrected for differences in sperm concentration. ( $n = 8$ )

Crosses	Hybrid Rate (%)	Hybrid Rate Expected	Hybridization Probability	$\chi^2$ Test (P)
C (♀) $\times$ [P (♂) + C (♂)]	10.9 $\pm$ 1.6	48.6	0.112	$P < 0.005$
P (♀) $\times$ [P (♂) + C (♂)]	5.7 $\pm$ 1.0	47.9	0.055	$P < 0.005$

1993). Instead, this study was designed to test whether this mating preference also exists in *C. farreri* and *P. yessoensis* and how much of the heterospecific sperm fertilization success was influenced at the presence of conspecific sperm.

The heterozygote deficiency obtained in these sperm competition crosses in the natural spawning condition may be explained by either (1) assortative fertilization (gamete choice); (2) differential fertilization (fertilization failure) or abortion. But the lack of heterozygote deficiency in the control crosses favors the assortative fertilization hypothesis because differential fertilization or abortion should have occurred both in control and sperm competition crosses. Moreover, abortion percentages seem insufficient to produce the observed heterozygote deficiency in the sperm competition crosses. Indeed, in the worst case, if we hypothesize that all the aborted embryos in our crosses were hybrids, then the hybrid rates won't reach the rate of expected ( $P < 0.05$ ) when the sperm use was random. Assortative fertilization must therefore have occurred in *C. farreri* and *P. yessoensis* with *C. farreri* eggs using average 89.1% of conspecific sperms and *P. yessoensis* eggs using average 94.3% of conspecific sperms in the control condition (temperature 17°C and salinity 31.5‰). These are consistent with those of other marine invertebrates, which have recently been investigated. In sea urchins of *Echinometra oblonga* and *E. sp.C.*, an average of 87.8% *E. sp.C.* eggs were fertilized by conspecific sperms when exposed to equal amount sperm of both species (Geyer & Palumbi 2005). In the mussels *Mytilus edulis* and *M. galoprovincialis*, the total conspecific sperm use of both females was 77% when all four gametes were mixed together (Bierne et al. 2002). Conspecific sperm precedence in free spawning invertebrates shows that the simple surfaces of eggs and sperm provide ample opportunity for egg choice and sperm competition even in the absence of intricate behavior or complex reproductive morphologies.

Mechanistically, to date, only few attempts were made to investigate the mechanism of sperm biased use in free-spawning invertebrates. In sea urchins, Metz et al. (1994) demonstrated that

the specificity of fertilization between *E. oblonga* and *E. mathaei* is controlled by the interaction of the sperm protein bindin with a receptor on the egg surface. Palumbi (1998, 1999) further elucidated that different bindin alleles confer different fertilization properties in free spawning sea urchin *Echinometra mathaei*, and fertilization differences reflect male bindin genotype and are under the control of both female and male genotypes. Unfortunately, till now no detailed compositional or functional studies on sperm protein bindin were carried out in scallops, but a similar cell-cell interaction mechanism may, as well, be functioning in the assortative mating system in *Chlamys farreri* and *Patinopecten yessoensis*; if this were true, then the fact that the assortative fertilization can be influenced by environmental factors may provide another clue and possibility to further understanding of the mechanism behind the mating preference system. As shown in this study, the mean hybrid rate obtained in these crosses varied from 0.5% to 20.0% as temperature and salinity changed. It may suggest that the egg-sperm recognizing system may be slightly disturbed when fertilization condition changes. What event may take place in this recognizing system when the environmental factors change and what elements on the gamete surface may be involved in this process are now of great interest to us. But alternatively, it may just be the byproduct of different sperm tolerance under different conditions. Because the different sperm tolerance of the two species may influence the practical relative sperm concentration when the sperm competition took place. Whatever it is, assortative fertilization seemed to occur despite the temperature and salinity. Because even the highest mean hybrid rate of 20.0% at the temperature of 11°C was still likely to be the result of assortative fertilization in consideration of the same concentration of conspecific and heterospecific sperm added. Unfortunately, as no control data of hatching rate was available in each cross under these changed conditions, we can't speculate further.

Although much remains unknown about the underlying mechanism of assortative mating system until now, the phenomenon itself has much implication for our practical hybrid production. In

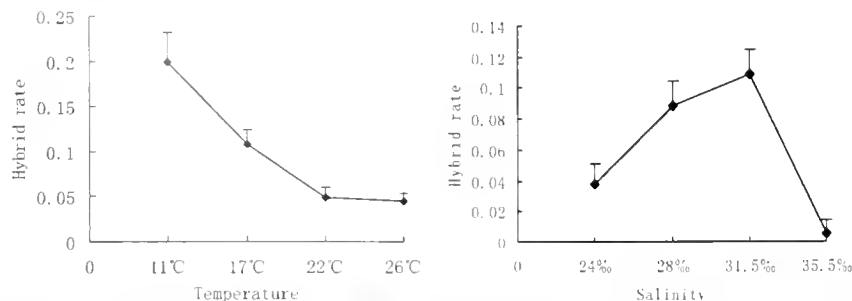


Figure 2. The mean hybrid rate in each sperm competition cross of *Chlamys farreri* (♀)  $\times$  [*Patinopecten yessoensis* (♂) + *Chlamys farreri* (♂)] in different conditions ( $n = 8$ ).

TABLE 3.

The mean hybrid rate ( $\pm$ SE) of sperm competition crosses in different conditions. The percentages were compared with random expectation by a  $\chi^2$  test of the observed number of hybrid offspring in each condition versus the number observed in natural spawning condition ( $n = 8$ ).

Environmental Factors	Level Set	Hybrid Rate (%)
Temperature	11 C	20.0 $\pm$ 3.2 <sup>b</sup>
	17 C	10.9 $\pm$ 1.6 <sup>d</sup>
	22 C	4.9 $\pm$ 1.1 <sup>e</sup>
	26 C	4.5 $\pm$ 0.9 <sup>e</sup>
Salinity	24.0‰	3.8 $\pm$ 1.3 <sup>b</sup>
	28.0‰	8.8 $\pm$ 1.7 <sup>a</sup>
	31.5‰	10.9 $\pm$ 1.6 <sup>d</sup>
	35.5‰	0.5 $\pm$ 0.9 <sup>b</sup>

<sup>a-e</sup> Means within the same factor, treatments sharing a common superscript letter in the same column were not significantly different ( $P > 0.05$ ) as determined by  $\chi^2$  test.

the hybridization of *Chlamys farreri* and *Patinopecten yessoensis*, especially in a large scale breeding program, conspecific sperm contamination can cause problems that affect the efficiency of hybridization. The contamination can come from many sources such as: careless handling of fishery tools, sperm from natural seawater via water supply system and a small proportion of her-

maphroditic individuals in scallops. In some cases, the heterospecific sperm was added prior to the female spawning with the intention to decrease the chance of self fertilization. But it commonly results in little amelioration. From the present study, we know that the preaddition of heterospecific sperm before spawning won't necessarily decrease the self fertilization rate because of the conspecific sperm precedence. So more care, such as parent scallops selection or more careful handling of fishery tools, should be taken prior to or during the hybridization to minimize the sperm contamination. Another implication we can get from the study is that, because environmental factors can affect the assortative fertilization in the competitive fertilization system, some measurements, such as fertilization at lower temperatures, fertilization in a optimum water salinity and pH value can be taken to increase the hybrid rate in hybridization, if contamination cannot be avoided.

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## GENOTYPE-DEPENDENT SURVIVAL, GROWTH, AND PRODUCTION IN CULTURED BLUE MUSSELS, *MYTILUS* SPP.: RESULTS OF A RECIPROCAL SEED TRANSFER EXPERIMENT

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**ABSTRACT** A reciprocal mussel seed transfer experiment was conducted involving two farm sites (Charles Arm and Thwart Island) whose seedstocks differed significantly in their respective compositions of *Mytilus edulis* L., *M. trossulus* Gould and hybrids. Electrophoretic variation at the mannose phosphate isomerase (*Mpi*) enzyme locus was used to classify the two species and their hybrids. Survival and growth in shell length, wet weight, shell weight and flesh weight were monitored over a 14-mo period. Both stock groups originating from Thwart Island seed (TI reared on its native site and the transferred TI-X stock) and the transferred stock group originating from Charles Arm seed (CA-X) showed no evidence of genotype-dependent variability in survival whereas, in the Charles Arm seed reared on its native site (CA), the proportion of *M. edulis* declined in relation to *M. trossulus*. There was no evidence of either a survival advantage or disadvantage in hybrids. After 14 mo, both transferred stocks (CA-X and TI-X) had comparatively higher frequencies of *M. edulis* and hybrids and lower frequencies of *M. trossulus* than did their respective nontransferred stocks (CA and TI) indicating a significant genotype  $\times$  site interaction effect on survival. Significant species, stock, stock  $\times$  time, species  $\times$  time and stock  $\times$  species  $\times$  time effects on shell length, wet weight, shell weight and flesh weight occurred. Intrastock growth rates of *M. trossulus* were lower than *M. edulis* and hybrids for shell length (3 of 4 stocks), and for the 3 weight variables, wet weight, shell weight and flesh weight (4 of 4 stocks). These results support the conclusion that growth is genotype-dependent in rope-cultured mussel populations and that these differences in weight growth favoring *M. edulis* are maintained when such stocks are transferred to other sites. Mussel biomass ( $\text{kg m}^{-2}$ ) was similar for the CA, CA-X and TI-X stock groups and lower for the TI stock group. Rates of production (wet weight) over the 14-mo growout period were similar for the CA, CA-X and TI stock groups and significantly higher for the TI-X stock group. In both stock groups originating from Thwart Island seed (TI reared on its native site and the transferred TI-X stock), the intrastock rates of production of *M. edulis* and hybrids were significantly greater than sympatric *M. trossulus*. However, in the transferred Charles Arm stock (CA-X), the rates of production were similar in *M. edulis* and *M. trossulus* and significantly greater in hybrids. In the Charles Arm stock reared on its native site (CA), the rate of production of *M. trossulus* was significantly greater than either *M. edulis* or hybrids. We conclude *M. edulis* and hybrids have intrinsically greater rates of weight growth, but not necessarily length growth, than does *M. trossulus*. Neither *M. edulis* nor *M. trossulus* nor hybrids have consistently greater rates of survival or production in suspended rope culture. These results are discussed in the context of a directed seed-stocking program involving the transfer of *M. edulis* seedstocks to sites whose native stocks have a high *M. trossulus* component as a means to enhance commercial mussel production within the *M. edulis*-*M. trossulus* hybridization zone in Atlantic Canada.

**KEY WORDS:** *Mytilus*, genotype-dependent, reciprocal transfer, survival, growth, production

### INTRODUCTION

Two mytilid mussel species, *Mytilus edulis* and *M. trossulus*, are endemic to northeastern North America. They are sympatric throughout a broad overlapping distributional zone extending from Newfoundland through the Gulf of St. Lawrence, along the Atlantic coast of Nova Scotia and into the northeastern United States (McDonald et al. 1991, Mallet & Carver 1992, Bates & Innes 1995, Penney & Hart 1999, Rawson et al. 2001). Localized mussel populations are typically composed of mixtures of the two species, often varying widely in relative frequency between adjacent sites in close proximity to each other (Mallet & Carver 1992, Bates & Innes 1995, Penney & Hart 1999). Indeed, nearby sites often display intersite genetic heterogeneity on a magnitude scale comparable to sites much farther apart (Penney & Hart 1999). The two species also readily hybridize (Saavedra et al. 1996, Comesaña et al. 1999, Penney & Hart 2002a, Penney et al. 2002) forming a highly diverse, genetically heterogeneous hybridization zone.

Survival is genotype-dependent in rope-cultured populations on farm sites within the hybridization zone (Penney & Hart 2002a). Thus, temporal genetic heterogeneity is likely a significant contributing factor to the extensive genetic population structuring found throughout the hybridization zone (Penney & Hart 1999). Furthermore, growth in shell length, wet weight, shell weight and

flesh weight are also genotype-dependent with growth rates for these weight variables being greater in *M. edulis* than *M. trossulus*, whereas growth rates in hybrids are typically intermediate between the two (Penney et al. 2002). Thus, genotype-dependent processes may be a significant contributory factor influencing production variability among mussel farms (Penney & Hart 2002a). Currently, many mussel farms within the hybrid zone, particularly sites in Newfoundland, use seed collected from their native sites with little regard or knowledge of their species composition. Because the relative species composition of the native seedstocks at these sites may differ dramatically (Penney & Hart 1999), this practice may contribute to the highly variable intersite growth and production indices throughout the industry. A recommendation has been made that growout of transplanted seed derived from unspecific or high-ratio *M. edulis* stocks should increase farm site production and reduce intersite variability in comparison with the use of seed collected on-site from indigenous mixed-species stocks (Penney et al. 2002). The greatest improvement in production would be anticipated at sites whose native seedstocks are primarily *M. trossulus*.

However, this recommendation has been advanced as a result of work carried out with indigenous seedstocks reared on their native sites. As Penney et al. (2002) pointed out, concern exists that our present state of knowledge of relationships among genetic heterogeneity, environmental variability and the intrinsic range of adaptive physiological flexibility in mussels (Hawkins & Bayne

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1992) is inadequate to reliably predict the subsequent production performance of transplanted seedstocks. Yet, achieving predictable results from intersite seed transfers is key to successful implementation of a directed seed transplantation program by industry to increase commercial production. Implicit in the above recommendation are the assumptions that (a) mussel performance indices, which control production (e.g., survival and growth) are significantly controlled by stock genetics and thus are predictable when transferred among sites and (b) the range of natural intersite environmental variability over geographic scales wherein intersite stock transfers are practically feasible to industry does not exceed the intrinsic scope of adaptive flexibility within mussel populations.

In the various mussel species being commercially cultivated, production indices are affected by variable growth and survival, both of which are known to be significantly influenced by stock-related factors (i.e., genetic variability; Fuentes et al. 1992, Stirling & Okumus 1995, Mallet & Carver 1995, Myrand & Gaudreault 1995 and site-related environmental variability; Dickie et al. 1984, Mallet & Carver 1989, Mallet et al. 1987). However, supporting evidence is somewhat equivocal concerning the comparative survival and growth of mussel stocks when transplanted versus when reared indigenously. There is some supporting evidence that interstock mortality differences are maintained when mussel stocks are transferred to new sites (Mallet et al. 1990, Myrand & Gaudreault 1995) suggesting that among-site survival variation is more influenced by stock genetics than environmental factors. In a series of experimental stock transfers in Nova Scotia, Mallet et al. (1990) noted survival rates in several stocks were unaffected by transfer leading to their conclusion that these stocks seemed insensitive to the environmental gradient. However, Dickie et al. (1984), Mallet et al. (1987) and Mallet & Carver (1989) concluded variation in mussel growth rates were mainly attributable to site-related environmental effects rather than stock (genetic) effects.

On a practical level, the mussel culture industry based in Prince Edward Island has successfully transplanted mussel seed from designated seed production sites to other growout sites over geographic scales of several kilometers. However, in Prince Edward Island, both the donor and recipient sites have indigenous mussel stocks that are virtually unispecific *M. edulis* (Mallet & Carver 1999). Similarly, for other mussel species and locations worldwide where commercial industry routinely transfers seedstocks successfully, (e.g., *M. galloprovincialis*) in Spain (Fuentes et al. 1994) and *Perna canaliculatus* in New Zealand (Alfaro & Jeffs 2003), these are areas with relatively low interstock genetic diversity. However, within the Atlantic Canadian hybrid zone, it has previously been observed that pure *M. edulis* stocks transferred from different locations can vary substantially in terms of tissue and shell growth, mortality and production (Mallet & Carver 1999). Past stock transfers involving more genetically heterogeneous seed populations have sometimes yielded unexpected growth and mortality patterns (Mallet & Carver 1989; Johannesson et al. 1990, Kautsky et al. 1990), which would be highly undesirable in a commercial context. Mallet and Carver (1999) have noted that transferred pure *M. edulis* stocks may not necessarily outperform a local mixed-species stock suggesting the possibility of significant localized adaptations in resident stocks. Interpretation of these sometimes conflicting results has been complicated by inadequate knowledge of stock genetic composition leading to concern regarding the predictability of subsequent survival and growth indices in transplanted seedstocks. This is especially so when the candidate seed-

stock for transplanting is genetically quite different from the indigenous stock (e.g., a unispecific *M. edulis* seedstock when transplanted to a predominately *M. trossulus* site). Because high-ratio *M. trossulus* populations are very common in most areas outside the general vicinity of Prince Edward Island, this concern is of significant importance to further industry development in the Atlantic Canadian hybrid zone.

In this study, we attempt to address this issue. We have conducted a reciprocal transfer experiment involving two genetically heterogeneous seedstocks, one a high ratio *M. edulis* stock and the other a relatively higher ratio *M. trossulus* stock from within the *M. edulis*-*M. trossulus* hybridization zone in Notre Dame Bay, Newfoundland. A suitable unispecific *M. edulis* stock was unavailable for intersite transfers within our geographic area under current seedstock transfer regulations, so we had to content ourselves with a known local high-ratio *M. edulis* stock. We compare the growth, survival and production patterns of each stock and of the *M. edulis*, *M. trossulus*, and hybrid constituents within each of the resulting four stocks (two reared indigenously, two reciprocally transferred) over a 14-mo period of rope culture at two farm sites. Specifically, we test the hypothesis that growth, survival and production rates of *M. edulis* and possibly hybrids as well are intrinsically higher than sympatric *M. trossulus* and that these intrastock differences are maintained when stocks are transplanted to other sites. The results are used to determine whether the substitution of indigenous *M. trossulus* seedstocks through intersite transfer of predominately *M. edulis* seedstock for growout can be supported as a viable approach to enhance commercial farm production.

## METHODS

Previously, mussel populations at two farm sites in Notre Dame Bay (Charles Arm and Thwart Island), Newfoundland, were identified as predominately *M. edulis* and *M. trossulus* respectively (Penney & Hart 2002a). In August 2000, a reciprocal transfer experiment was set up involving seed collected on ropes suspended subtidally at both sites. Four seed groups were monitored for survival and growth over a 14-mo period: Charles Arm seed kept at Charles Arm (CA), Charles Arm seed transferred to Thwart Island (CA-X), Thwart Island seed kept at Thwart Island (TI), and Thwart Island seed transferred to Charles Arm (TI-X). Seed mussels from each stock were mechanically de-clumped to ensure randomization and then sleeved in standard commercial plastic mesh using current industry husbandry practices for rope culture of blue mussels. Seed mussel sleeves from each donor stock were then either placed in the water at Charles Arm or transferred to Thwart Island. Each sleeve was 1 m in length and was vertically suspended at 0.5 m intervals from horizontal mainline ropes with flotation sufficient to ensure stable suspension at depths of approximately 4–6 m.

Three replicate sleeves were taken from each donor seed stock for laboratory analysis at the time of sleeving. On subsequent sample dates (November 2000, May 2001 and October 2001), three replicate sleeves were retrieved from each of the four stock groups on their respective farm sites. All the mussels were removed from measured sections of each sleeve and counted. On each sample date, one hundred individuals were randomly selected from each sleeve per stock group and measured for shell length, drained wet weight, shell weight and flesh weight (total of 300 from each site  $\times$  group combination). Shell length was measured with digital callipers (Mitutoyo, Aurora, IL, USA). Wet weights

were obtained after opening the shell valves and allowing the shell cavity to drain on paper towel. Hepatopancreas tissue was excised from each selected mussel, lyophilized and stored at 5°C for later allozyme analysis. The remaining soft tissues were then removed and shell weights were obtained after drying overnight. Flesh weights were calculated by difference between wet weight and dry shell weight.

For allozyme analysis of mannose phosphate isomerase (*Mpi*, EC 5.3.1.8), a small amount of freeze-dried material was ground to a fine powder with 0.5 M Tris HCL pH 8.0 buffer containing 20% glycerol and 0.2% NAD. Subsequent electrophoresis and staining on cellulose acetate plates followed the general procedure of Herbert and Beaton (1989) for the mannose phosphate isomerase locus, although a constant current of 2 mA per plate was used during the electrophoretic runs. Allele nomenclature is similar to that used by previous authors (Koehn et al. 1984, McDonald & Koehn 1988). Individual mussels were classified as *M. edulis*, *M. trossulus* or their hybrids based on their *Mpi* electrophoretic patterns according to previously published references for North American populations (Varvio et al. 1988, McDonald et al. 1991).

Survival over time was calculated as the difference in mean number of mussels sleeve<sup>-1</sup> on each sample date compared with the initial sample at the time of sleeving. In this context, changes in survival do not distinguish between changes caused by mortality versus dropoff from the culture gear. Rate of production was defined as the rate of change in wet weight between the initial and final sleeve weights where initial and final sleeve weights were calculated as the mean individual mussel wet weight multiplied by the number of mussels present. Rate of production was then calculated from the formula: Production = (final wet weight - initial wet weight)/initial wet weight. All subsequent statistical analyses were run with the SAS software system (SAS Institute Inc. 1988). Interstock and intergenotype differences in initial frequency distributions and survival were compared with the chi-square statistic. ANOVA were used to compare interstock and intergenotype differences in initial size of seed mussels and production over time. Tukey tests were applied to determine which comparisons were statistically significant. ANCOVA (homogeneity of slopes models) were used to compare interstock and intergenotype differences in growth.

## RESULTS

### Survival

In August 2000, mussel seed from the two donor sites, Charles Arm (CA) and Thwart Island (TI), were sleeved volumetrically after de-clumping to ensure randomization of all stock. The initial seed sleeving density was 2.441 m<sup>-1</sup> and 2.471 m<sup>-1</sup> for CA and TI seedstocks respectively. These differences were not significant (Student's *t*-test,  $P > 0.05$ ). The frequency distribution of *M. edulis*, *M. trossulus* and their hybrids differed significantly in the two seedstocks at the time of sleeving. The initial CA seedstock was comprised of 76.2% *M. edulis*, 7.4% *M. trossulus* and 16.4% hybrids. In comparison, the initial TI seedstock was 54.7% *M. edulis*, 26.7% *M. trossulus* and 18.6% hybrids ( $\chi^2$ ,  $P < 0.001$ ) (Fig. 1). Over the 14-mo growout period, genotype-dependent survival patterns became evident in the CA stock but not in the CA-X, TI or TI-X stocks (Fig. 2). In the CA stock, the frequencies of *M. edulis* and hybrids declined to 65.9% and 14.7% respectively, whereas the frequency of *M. trossulus* increased to 19.4% ( $\chi^2$ ,  $P < 0.001$ ). After 14 mo, the frequencies of *M. edulis*, *M. trossulus* and their

hybrids were 70.2%, 20.4% and 9.4% in the CA-X stock; 48.3%, 35.0% and 16.7% in the TI stock and 57.5%, 23.1%, and 19.4% in the TI-X stock respectively. However, these frequency changes of *Mytilus* genotypes over the 14-mo growout period in the CA-X, TI and TI-X stocks were not significant compared with their respective initial Charles Arm or Thwart Island seedstock ( $\chi^2$ ,  $P > 0.05$ ).

Mean survival over the 14-mo growout period was significantly lower in the transferred Thwart Island stock (TI-X, 32.6%) than for either of the stocks derived from Charles Arm seed (nontransferred CA, 43.7%; transferred CA-X, 46.8%) or the nontransferred stock from Thwart Island seed (TI, 48.1%) (Fig. 2). Interstock differences in mean survival were not significant among the CA, CA-X and TI stocks (ANOVA,  $P > 0.05$ ). Comparison of the genotypic frequency distributions of the CA versus CA-X stocks and the TI versus TI-X stocks after 14 mo gave evidence of a significant site effect on the survival of the respective *Mytilus* genotypes. After 14 mo, the genotypic frequency distributions of the CA and CA-X stocks had significantly diverged from each other ( $\chi^2$ ,  $P < 0.001$ ) and the TI and TI-X stocks were also significantly different ( $\chi^2$ ,  $P < 0.006$ ). In both cases, the transferred stocks (CA-X and TI-X) had comparatively higher frequencies of *M. edulis* and hybrids and lower frequencies of *M. trossulus* than did their respective nontransferred stocks (CA and TI). This provides evidence of a significant site  $\times$  genotype interaction effect on survival among the three *Mytilus* genotypes.

### Growth

When sleeved in August 2000, mean shell length, wet weight, shell weight and flesh weight of seedstocks from both donor sites differed significantly (Tukey,  $P < 0.05$ ) (Table 1). For all four variables, CA seed mussels were larger than the TI mussels. Within the CA seedstock, there was no evidence of species-related differences in either of the four variables. However, within the TI seedstock, *M. trossulus* mussels were significantly larger than *M. edulis* and hybrids in terms of shell length, wet weight and flesh weight, but interspecies differences in shell weight were not significantly different.

Variability in all 4 growth variables (shell length, wet weight, shell weight and flesh weight) were all significantly related to species, stock and growout time (ANOVA, Table 2). The interaction effects of species  $\times$  time and stock  $\times$  time were also significant for all four variables, whereas the interaction effect of stock  $\times$  species  $\times$  time was significant for the three weight variables but not shell length. The interaction term, species  $\times$  stock, was not significant for any of the four variables. The goodness of fit ( $R^2$ ) ranged from 0.66–0.72 for the four models.

Analysis of variance homogeneity of slopes models revealed that, over the 14-mo growout period, the two stocks raised at the Charles Arm site (CA and TI-X) consistently grew faster than did the two stocks reared at Thwart Island (TI and CA-X) (Fig. 3). At Charles Arm, the shell length slope for the transferred TI-X was significantly greater than the slope of the indigenous CA stock but slopes for all three weight variables were not significantly different between the two stocks. At Thwart Island, slopes for the transferred CA stock and the indigenous TI stock were not significantly different for shell length but, for all three weight variables, the respective slope for the CA-X stock was significantly greater than the TI stock.

Analysis of variance homogeneity of slopes models were subsequently used to elucidate the interaction effects involving spe-

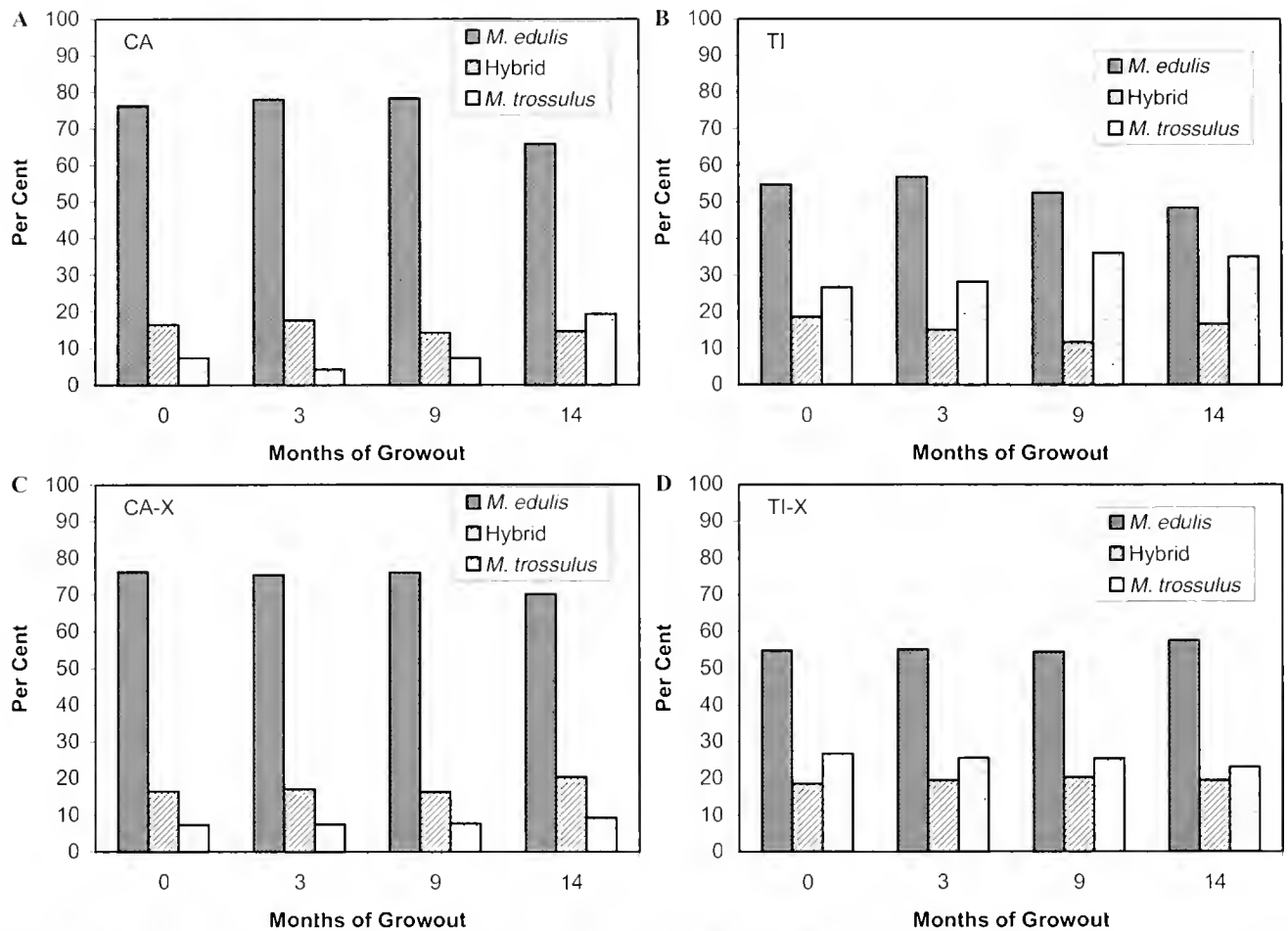


Figure 1. Mean species composition of each of the four stock groups (percent *M. edulis*, *M. trossulus*, and their hybrids) on four sampling dates during the 14-mo growout period determined from three replicate sleeves on each sample date.

cies within stocks over the 14-mo growout period. For shell length, within-stock model slopes for *M. trossulus* were significantly lower than both *M. edulis* and hybrids for all but the CA-X stock (Fig. 4). A consistent within-stock interspecific pattern emerged for all three weight variables. In each case, within-stock model slopes for wet weight (Fig. 5), shell weight (Fig. 6) and flesh weight (Fig. 7), were significantly lower for *M. trossulus* than for *M. edulis* and hybrids. For all four variables, slopes of *M. edulis* and hybrids were not significantly different from each other.

#### Production

After 14 months, the mussel biomass (mean wet weight  $\times$  number of survivors) of the CA CA-X and TI-X sleeves ranged from 4.26–4.49 kg m<sup>-2</sup> but were not significantly different (Table 3).

TABLE 1.

Initial size comparison of mussel seed from the two donor sites (Charles Arm, CA and Thwart Island, TI) at the time of sleeving: (a) full stock basis, and (b) and (c) by species within site. Numbers with different superscripts are significantly different within columns for each of (a), (b) and (c),  $P < 0.05$  (Tukey multiple range test).

Stock	Variable			
	Shell Length	Wet Weight	Shell Weight	Flesh Weight
(a)				
CA	26.42 <sup>a</sup>	1.28 <sup>a</sup>	0.45 <sup>a</sup>	0.82 <sup>a</sup>
TI	21.53 <sup>b</sup>	0.81 <sup>b</sup>	0.32 <sup>b</sup>	0.50 <sup>b</sup>
(b) CA				
<i>M. edulis</i>	26.29 <sup>a</sup>	1.27 <sup>a</sup>	0.45 <sup>a</sup>	0.82 <sup>a</sup>
Hybrid	26.30 <sup>a</sup>	1.24 <sup>a</sup>	0.44 <sup>a</sup>	0.80 <sup>a</sup>
<i>M. trossulus</i>	28.37 <sup>a</sup>	1.48 <sup>a</sup>	0.52 <sup>a</sup>	0.96 <sup>a</sup>
(c) TI				
<i>M. edulis</i>	21.07 <sup>a</sup>	0.76 <sup>a</sup>	0.31 <sup>a</sup>	0.45 <sup>a</sup>
Hybrid	20.53 <sup>a</sup>	0.67 <sup>a</sup>	0.27 <sup>a</sup>	0.41 <sup>a</sup>
<i>M. trossulus</i>	23.49 <sup>b</sup>	1.05 <sup>b</sup>	0.37 <sup>a</sup>	0.68 <sup>b</sup>

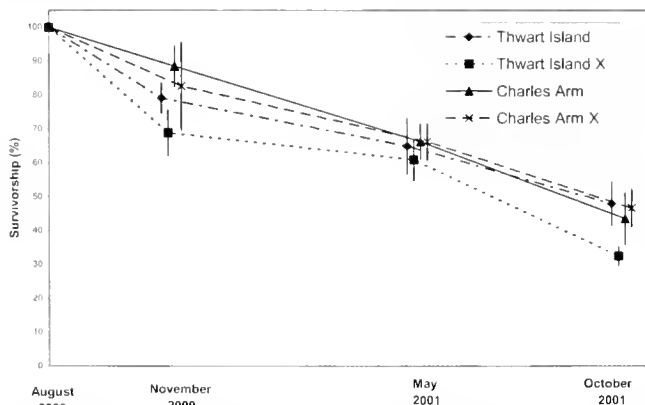


Figure 2. Comparative survival of the four stock groups over the 14-mo growout period. Plotted points are means  $\pm$  2 SE of counts on three replicate sleeves on each sample date.

TABLE 2.

Analysis of variance (ANOVA) test results for species (*M. edulis*, hybrid, and *M. trossulus*) and stock (CA, CA-X, TI, and TI-X) variation over the 14-month growout period for shell length, wet weight, shell weight, and flesh weight.

Variable	F Value							R <sup>2</sup>
	Species	Stock	Time	Sp. × Time <sup>‡</sup>	Sp. × Stk. <sup>‡</sup>	Stk. × Time <sup>‡</sup>	Stk. × Sp. × Time <sup>‡</sup>	
Shellen	7.76**	60.58***	5403.99***	25.81***	1.32	19.92***	1.99	0.69
Wetwgt	4.79**	15.42***	5257.92***	64.28***	0.96	75.71***	5.68***	0.70
Shellwgt	3.24*	11.08***	5479.08***	93.39***	0.74	96.29***	8.46***	0.72
Fleshwgt	5.65**	17.71***	4470.41***	38.21***	1.17	53.53***	3.45**	0.66

Numbers without superscript are not significantly different,  $P > 0.05$ ; \*\*\* $P < 0.0001$ ; \*\* $P < 0.01$ ; \* $P < 0.05$ . <sup>‡</sup>sp. = species, stk = stock, etc.

Mean mussel biomass of the TI stock group was significantly lower than either of the other three stock groups. The rate of production, defined as the increase in mussel wet weight  $m^{-1}$  of sleeve, was not significantly different among the CA, CA-X and TI stock groups but was significantly greater in the TI-X stock group (Table 3). Within both stock groups originating from the Thwart Island seedstock (TI and TI-X), *M. edulis* and hybrids had significantly greater production rates than *M. trossulus*. However, in the CA-X stock group, hybrids had a higher rate of production than either of the other two species. In the CA stock group, *M. trossulus*

had a significantly higher rate of production than either *M. edulis* or hybrids. Thus, neither *M. edulis* nor *M. trossulus* nor their hybrids consistently exhibit greater rates of production within all stock groups.

# DISCUSSION

Whether being considered from an individual farm or industry-wide perspective, the commercial industry objective is to maximize on-site production, while reducing production variability

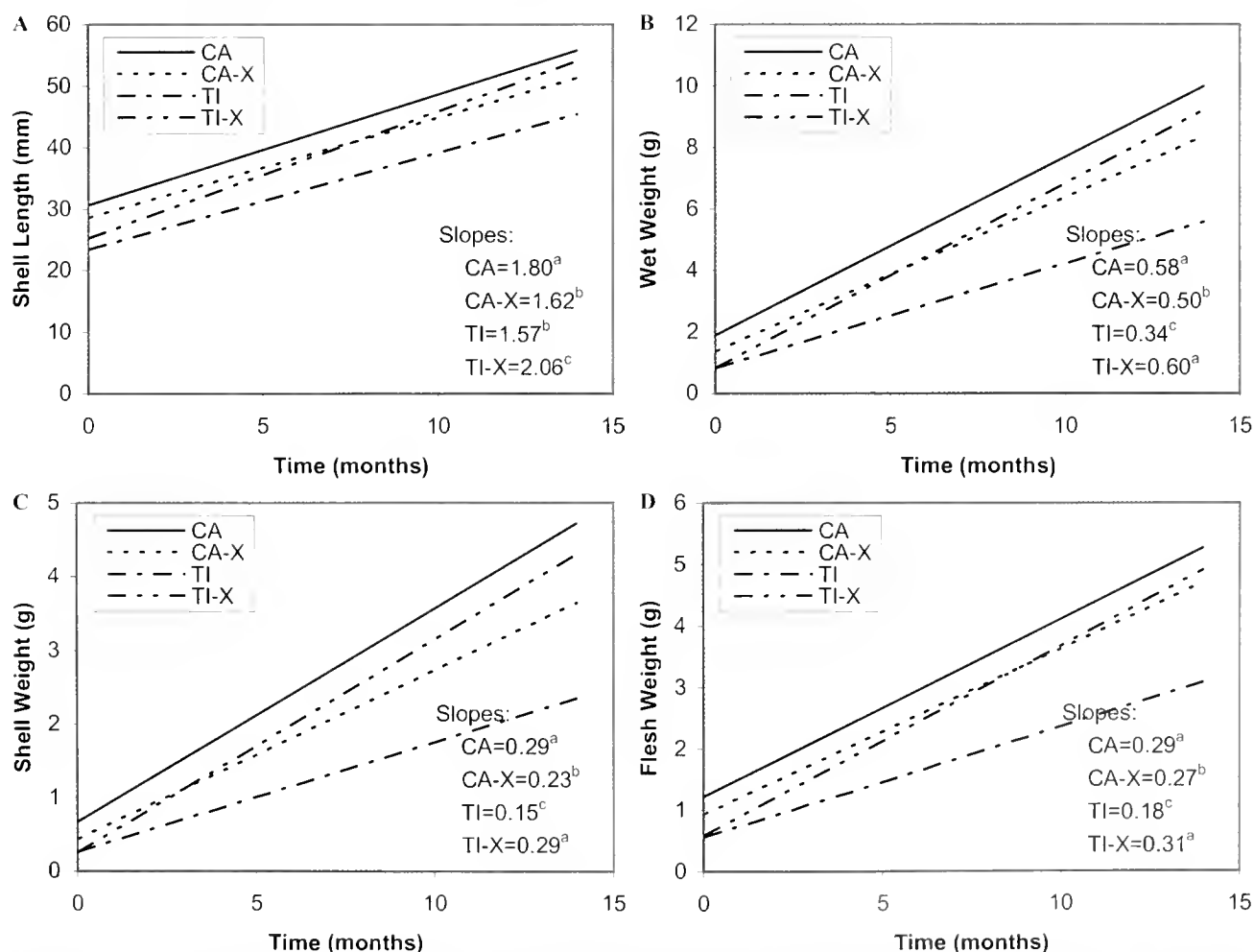


Figure 3. Comparative intersite growth in shell length, wet weight, shell weight, and flesh weight of each of the four stock groups over the 14-month growout period. Lines are fitted to the respective linear regression equations for each stock group over time. Different superscripts denote statistical significance between slopes (ANOVA homogeneity of slopes model,  $P < 0.05$ )

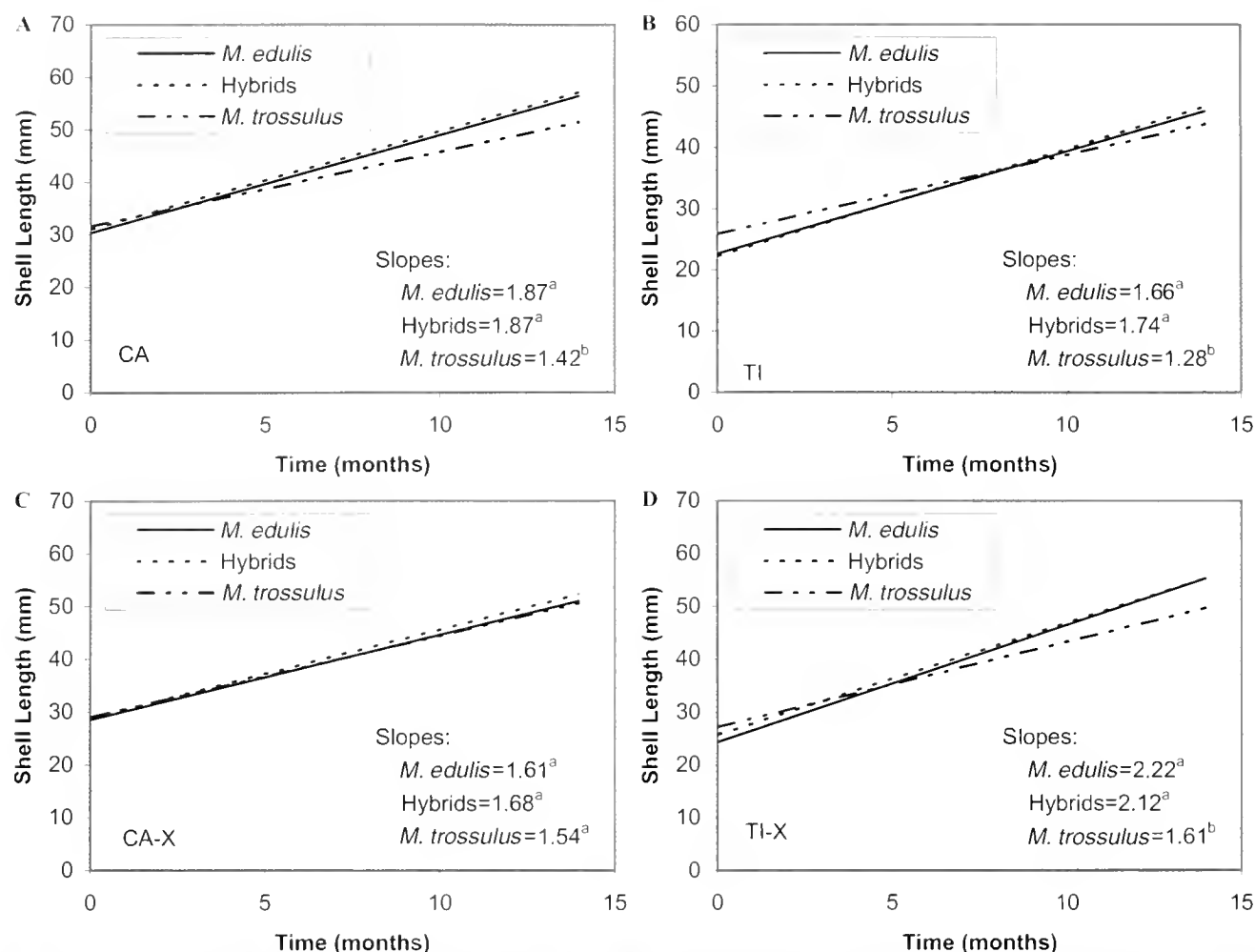


Figure 4. Comparative intrastock growth in shell length of *M. edulis*, *M. trossulus*, and their hybrids over the 14 mo growout period for each of the four stock groups. Lines are fitted to the respective linear regression equations of shell length over time for each stock group. Different superscripts denote statistical significance between slopes (ANOVA homogeneity of slopes model,  $P < 0.05$ ).

within and among farm sites. Genetic heterogeneity within and among sites can be a highly significant source of phenotypic variability in survival, growth and shell morphology (Penney & Hart 1999; Penney & Hart 2002a; Penney et al. 2002), all of which affect commercial industry production. At sites within mussel hybrid zones, genetic heterogeneity and phenotypic variability are both typically much greater than in areas occupied by single species (Gardner 1996). Because *M. edulis* exhibits faster growth in comparison with *M. trossulus* (Penney et al. 2002), it has been recommended that commercial production indices within the hybrid zone may be improved and intrasite and intersite variability reduced if industry were to convert to use of seed from unspecific or at least high-ratio *M. edulis* seedstock(s) replacing their present practice of seed collection from a multitude of sites, which often vary widely in species proportions (Penney et al. 2002). This would be especially beneficial at sites whose native mussel populations are predominately *M. trossulus*.

Commercial success of such a proposition depends on the relatively greater growth performance of *M. edulis* versus *M. trossulus* when stocks are grown on their native sites (Penney et al. 2002) being maintained when transplanted and for this growth differential to translate into increased production at the recipient site. The present work tests this proposition by assessing the relative

growth, survival and production dynamics of sympatric cohorts of these two species and their hybrids in a reciprocal transplant experiment. With respect to survival, we confirm earlier observations (Mallet & Carver 1995; Penney & Hart 2002a) that genotype-dependent survival patterns occur among the various *Mytilus* genotypes in rope-cultured mussel populations within the Atlantic Canadian hybrid zone. However, these patterns do not consistently favor survival of either *M. edulis* or *M. trossulus* or their hybrids at all sites. In other words, survival is influenced by a significant stock  $\times$  site interaction effect. It is also interesting to note that genotype-dependent selection favoring *M. trossulus* occurred in the Charles Arm stock reared on its native site. However, in a similar growout experiment involving an earlier year class of Charles Arm seed grown at the same site (Penney & Hart 2002a), there was no evidence of any selective survival pattern at the *Mpi* locus although overall survival of that Charles Arm year class was lower than the Thwart Island stock at that time. We infer from these results that genotype-dependent survival patterns are also temporally variable among year classes within the same stock, possibly indicative of a genotype-dependent interaction with changing environmental conditions. Thus, in a commercial context, year-class to year-class survival patterns within rope-cultured mussel populations are currently somewhat unpredictable. Because

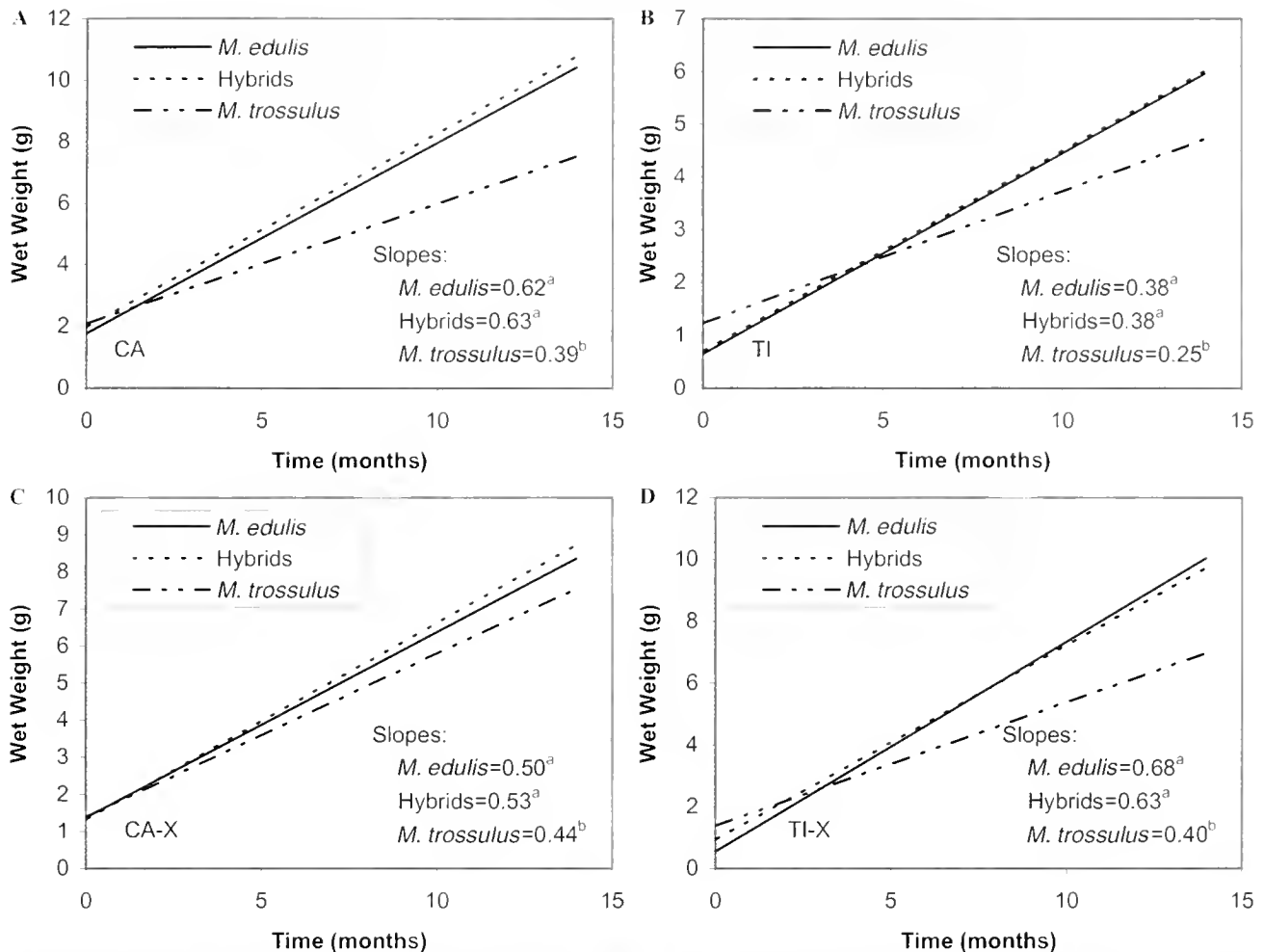


Figure 5. Comparative intrastock growth in wet weight of *M. edulis*, *M. trossulus*, and their hybrids over the 14 mo growout period for each of the four stock groups. Lines are fitted to the respective linear regression equations of wet weight over time for each stock group. Different superscripts denote statistical significance between slopes (ANOVA homogeneity of slopes model,  $P < 0.05$ ).

variable survival patterns may strongly affect production rates, this has important consequences for mussel aquaculture development within the hybrid zone.

The present results support earlier observations (Penney et al. 2002) that growth rates in wet weight, shell weight and flesh weight, but not necessarily growth in shell length, are typically greater in *M. edulis* and hybrids than in *M. trossulus*. We further conclude these genotype-dependent differences are maintained when stocks are transplanted to other sites. But, the intrinsically greater weight growth rates of *M. edulis* and hybrids do not necessarily translate into greater production performance in comparison with *M. trossulus*. Our analysis of production performance did not find either *M. edulis* or *M. trossulus*, or their hybrids consistently had comparatively higher rates of production in all cases. *M. edulis* and hybrid production rates were higher than *M. trossulus* in two of four stock groups, *M. edulis* and *M. trossulus* production rates were similar in a third group, and the *M. trossulus* production rate was greater than either of *M. edulis* or hybrids in the fourth group. The lower rates of production of *M. edulis* within the two stock groups originating from the Charles Arm seedstock (CA and CA-X) and the relatively high production rate of *M. trossulus* within the CA stock group was largely because of the relatively lower survival of *M. edulis* within these two groups and to the

relatively greater survival of *M. trossulus* in the CA stock group. The greater wet weight of the Charles Arm seed compared with Thwart Island seed at the time of sleeving also may have played a part in the comparatively lower production rates of their respective stock groups. Thus, we conclude our results do not support the proposition that *M. edulis* has intrinsically higher rates of production than *M. trossulus*. Therefore, a directed seed transfer program transplanting *M. edulis* stocks to sites whose native seedstocks are mostly *M. trossulus* will not necessarily result in increased rates of production on such mussel farms.

Lower survival rates of the *M. edulis* cohort compared with *M. trossulus* in mixed-species stocks has previously been reported from Lunenburg Bay in Nova Scotia (Mallet & Carver 1995). However, the relatively poor survival of *M. edulis* within stocks originating from Charles Arm seed is inexplicable at this point. This is especially so in light of the fact there was no evidence of genotype-dependent selection at the *Mpi* locus in a previous year class reared from seed to harvest size at Charles Arm using identical husbandry techniques and mussels of approximately the same age (Penney & Hart 2002a). *M. edulis* and *M. trossulus* are known to differ in their relative tolerance to temperature and salinity variations, with *M. trossulus* considered to be adapted to relatively colder, lower or more variable salinity environments (Väinölä &

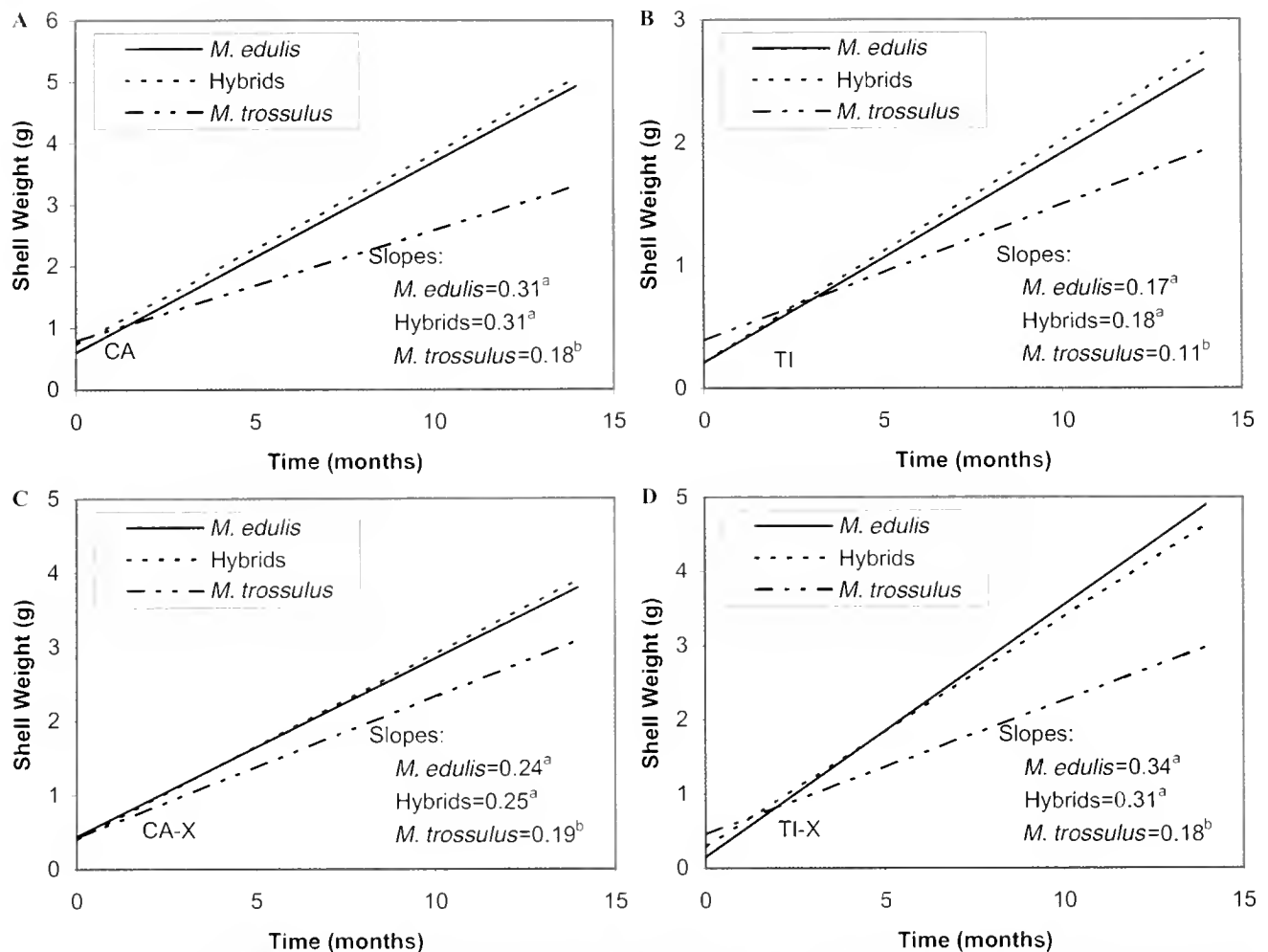


Figure 6. Comparative intrastock growth in shell weight of *M. edulis*, *M. trossulus*, and their hybrids over the 14 mo growout period for each of the four stock groups. Lines are fitted to the respective linear regression equations of shell weight over time for each stock group. Different superscripts denote statistical significance between slopes (ANOVA homogeneity of slopes model,  $P < 0.05$ ).

Hvilson 1991, Mallet & Carver 1995, Rawson et al. 2001). However, it should be emphasized at this point that, although the transfer of stocks across large environmental gradients is known to have potentially significant deleterious effects on mussel survival (Theisen 1978, Kautsky et al. 1990, Johannesson et al. 1990), the two sites used in the present work are both located in the same bay and subjected to similar thermal and salinity regimes. Thus, no obvious strong environmental gradient exists between the Thwart Island and Charles Arm sites, which might offer a likely explanation for the results of the present study.

Genotype-dependent mortality has previously been reported in mixed *M. edulis*-*M. trossulus* stocks (Pedersen et al. 2000) and temporal and/or stock-related variability in its expression may be commonplace within Atlantic Canadian mussel stocks. This is evidenced by a reciprocal seed transfer experiment by Myrand and Gaudreault (1995) who found that four mussel stocks originating from nearby sites in the Magdalen Islands differed dramatically in resistance to summer mortality despite all being predominately *M. edulis* (Tremblay et al. 1998). In a subsequent transfer experiment, it was noted the resistant stock suffered a significant decrease in degree of heterozygosity when transferred to other nearby sites leading to the hypothesis that more heterozygous individuals migrate to the outer regions of sleeves faster than more homozygous

individuals where they are more prone to drop-off caused by turbulence (Tremblay et al. 1998). However, Penney and Hart (2002a) evaluated multilocus heterozygosity at five of the same loci used by Tremblay et al. (1998) and found no evidence of temporal changes in heterozygosity in rope-cultured populations of either the Charles Arm or Thwart Island stocks. Thus, similar heterozygosity-fitness relationships in the Charles Arm stock appear an unlikely explanation for the relatively poor survival of the *M. edulis* component at Charles Arm.

Clearly, some factor other than simply genotypic variability at the *Mpi* locus results in significant intrastock and temporal survival variability in mixed *M. edulis*-*M. trossulus* stocks, although that unknown factor does seem linked to allelic variability at the *Mpi* locus. Allelic variability at several allozyme loci are known to be correlated with *Mpi* allelic variability in North American mussels (Varvio et al. 1988, McDonald et al. 1991) including native stocks at the two sites used in the present work (Penney & Hart 1999). Genotype-dependent selection at one such locus, *Gpi*, favoring survival of electrophoretically slower alleles has previously been noted in rope-cultured mussels from both Charles Arm and Thwart Island (Penney & Hart 2002a). Based on experimental work showing the enzyme products of different *Gpi* alleles had different thermal optima (Hoffman 1984, Hall 1985), Penney and



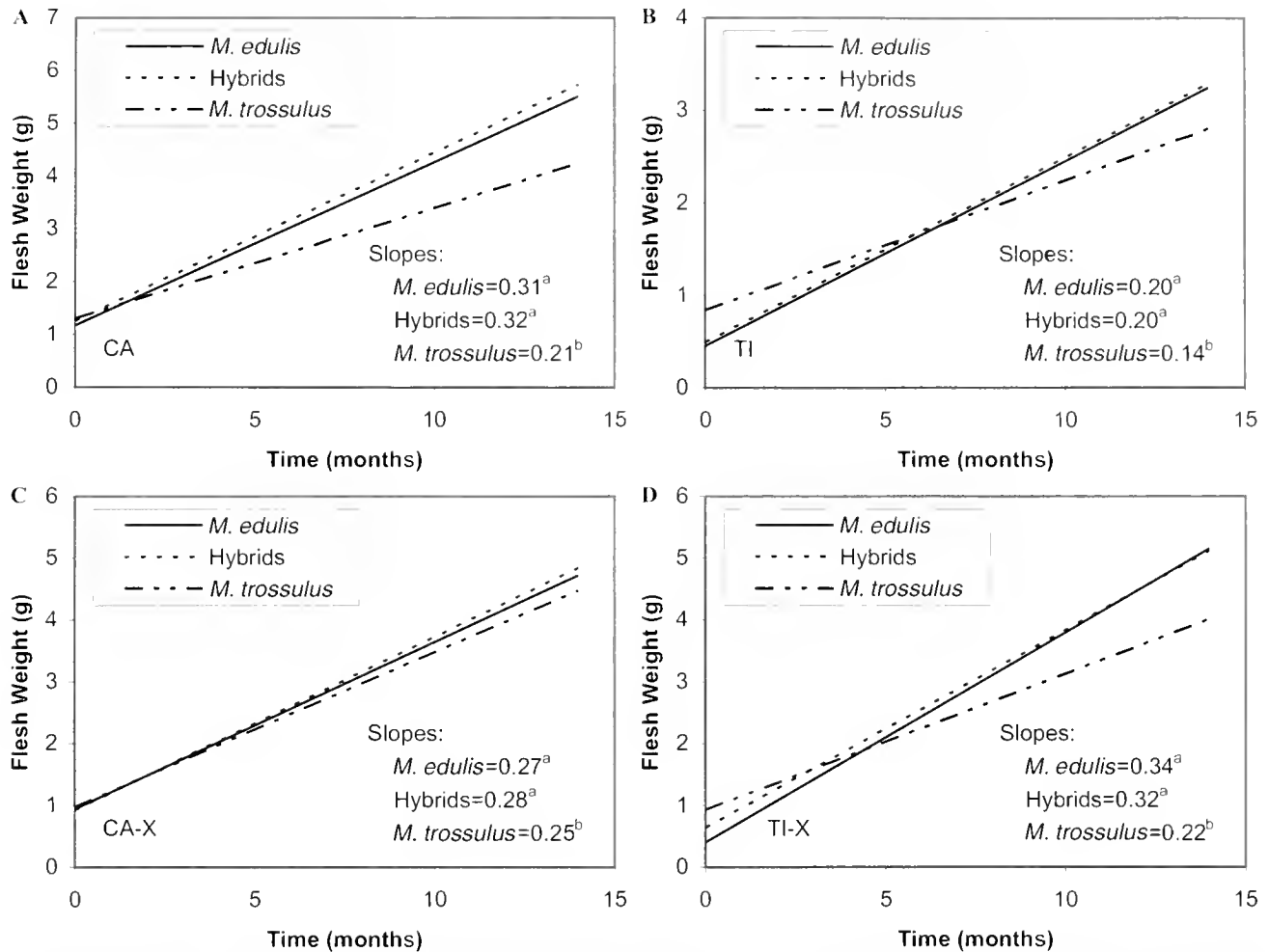


Figure 7. Comparative intrastock growth in flesh weight of *M. edulis*, *M. trossulus*, and their hybrids over the 14 mo growout period for each of the four stock groups. Lines are fitted to the respective linear regression equations of flesh weight over time for each stock group. Different superscripts denote statistical significance between slopes (ANOVA homogeneity of slopes model,  $P < 0.05$ ).

Hart (2002a) advanced the hypothesis that suspended mussel culture may selectively favor individuals with electrophoretically slower *Gpi* alleles, whereas individuals with electrophoretically faster alleles would have an advantage in intertidal shore populations exposed to high air temperatures during emersion.

Electrophoretically slower *Gpi* alleles are associated more with *M. trossulus* than with *M. edulis* (Varvio et al. 1988, McDonald et al. 1991, Penney & Hart 1999), and the incidence of electropho-

retically faster alleles is higher in the native Charles Arm stock than in Thwart Island stock. This could offer a potential physiological explanation for the relatively lower survival of the stocks originating from Charles Arm seed. While purely speculative, the hypothesis that slower *Gpi* electromorphs may be favored in subtidal conditions is indirectly supported by comparison of *Mytilus Gpi* electromorphs with those of the horse mussel, *Modiolus modiolus*. The natural spatial distribution of the latter is subtidal

TABLE 3.

Initial sleeve weight (mean wet weight  $\times$  number<sup>-1</sup> of sleeve), final mussel biomass after 14 months growout (mean wet weight  $\times$  number<sup>-1</sup> surviving), and rate of production ((final weight - initial weight)/initial weight) of *M. edulis*, *M. trossulus*, hybrids, and all species combined in each of transferred and non-transferred stock groups. Numbers with letter and numeric superscripts are significant for each variable across rows or down columns respectively (Tukey,  $P < 0.05$ ).

Species	Initial Weight (kg m <sup>-1</sup> sleeve)		Mussel Biomass (kg m <sup>-1</sup> sleeve)				Rate of Production			
	CA & CA-X	TI & TI-X	CA	CA-X	TI	TI-X	CA	CA-TX	TI	TI-X
<i>M. edulis</i>	0.99	0.54	3.15	3.02	2.00	2.74	2.18 <sup>a1</sup>	2.05 <sup>a1</sup>	2.70 <sup>b1</sup>	4.06 <sup>c1</sup>
Hybrid	0.21	0.16	0.71	0.88	0.68	0.86	2.41 <sup>a1</sup>	3.24 <sup>b2</sup>	3.21 <sup>b2</sup>	4.31 <sup>c1</sup>
<i>M. trossulus</i>	0.11	0.37	0.63	0.36	1.09	0.71	4.64 <sup>a2</sup>	2.19 <sup>b1</sup>	1.98 <sup>b3</sup>	0.93 <sup>c2</sup>
All	1.31	1.07	4.49 <sup>a</sup>	4.26 <sup>a</sup>	3.77 <sup>b</sup>	4.31 <sup>a</sup>	2.43 <sup>a</sup>	2.25 <sup>a</sup>	2.53 <sup>a</sup>	3.03 <sup>b</sup>

(Minor 1950) and hence subjected to a colder, relatively narrow range of water temperatures than the typically intertidal distribution of North American mytilid populations. Whether coincidence or not, all 11 electromorphs identified at the *Gpi* locus in Newfoundland populations of *M. modiolus* are electrophoretically slower than those of neighboring mytilids (Penney & Hart 2002b).

In summary, the present work supports the conclusion that the relatively faster rates of weight growth in rope-cultured *M. edulis* and hybrids compared with *M. trossulus* exhibited on their native sites are maintained when transferred to other sites, at least insofar as the donor and recipient sites are not substantially different in environmental conditions. However, the relative survival of the two species and hybrids and hence their relative production performance do not appear predictable based on their *Mpi* genotype alone. This conclusion is consistent with that of Mallet and Carver (1999) who observed that several pure *M. edulis* stocks from Prince Edward Island, New Brunswick and Nova Scotia when

transferred to a site whose native population was primarily *M. trossulus* did not consistently outperform the local mixed-species stock. Therefore, selection of high performance seedstocks appropriate for widespread use by industry remains problematic and a need exists to conduct test transfers of each stock individually or, alternatively, greater understanding of the causal mechanisms of variable survival in mixed-species populations is required. However, because the greater shell weight of *M. edulis* may translate into lower levels of shell breakage during processing (Mallet & Carver 1995), selection of *M. edulis* stocks over local *M. trossulus* or mixed-species stocks for commercial growout appears likely to be a better alternative.

#### ACKNOWLEDGMENTS

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## GENETIC MANAGEMENT GUIDELINES FOR CAPTIVE PROPAGATION OF FRESHWATER MUSSELS (UNIONOIDEA)

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**ABSTRACT** Although the greatest global diversity of freshwater mussels (~300 species) resides in the United States, the superfamily Unionoidea is also the most imperiled taxon of animals in the nation. Thirty-five species are considered extinct, 70 species are listed as endangered or threatened, and approximately 100 more are species of conservation concern. To prevent additional species losses, biologists have developed methods for propagating juvenile mussels for release into the wild to restore or augment populations. Since 1997, mussel propagation facilities in the United States have released over 1 million juveniles of more than a dozen imperiled species, and survival of these juveniles in the wild has been documented. With the expectation of continued growth of these programs, agencies and facilities involved with mussel propagation must seriously consider the genetic implications of releasing captive-reared progeny. We propose 10 guidelines to help maintain the genetic resources of cultured and wild populations. Preservation of genetic diversity will require robust genetic analysis of source populations to define conservation units for valid species, subspecies, and unique populations. Hatchery protocols must be implemented that minimize risks of artificial selection and other genetic hazards affecting adaptive traits of progeny subsequently released to the wild. We advocate a pragmatic, adaptive approach to species recovery that incorporates the principles of conservation genetics into breeding programs, and prioritizes the immediate demographic needs of critically endangered mussel species.

**KEY WORDS:** freshwater mussels, genetic guidelines, conservation units, artificial propagation, imperiled species.

### INTRODUCTION

North America contains the greatest diversity of freshwater mussels in the world, approximately 300 species. However, the superfamily Unionoidea is the most imperiled group of animals in the United States, with 213 species (72%) considered endangered, threatened, or of special concern (Williams et al. 1993, Neves 1999). Already, approximately 35 species, or 12% of the North American mussel fauna, have become extinct in the last 100 y (Neves et al. 1997), an extinction rate comparable to estimated faunal losses in tropical rainforests (Ricciardi & Rasmussen 1999). For example, the Tennessee River basin historically was home to 102 species of mussels, and hence is the putative center of mussel diversity in North America (Parmalee & Bogan 1998). Of those original 102 species, 12 are extinct, 26 are listed as endangered under the Endangered Species Act, 20 are extirpated from the basin, and only about 30 species have stable populations (Parmalee & Bogan 1998). Most of the endangerment is caused by habitat loss and degradation caused by dams, sedimentation, water pollution, dredging and other anthropogenic factors (Neves et al. 1997, Neves 1999). Without immediate efforts to recover the 70 federally listed and numerous other imperiled species in United States watersheds, the extinction of additional species is likely. With this goal in mind, a committee of experts prepared a National Strategy for the Conservation of Native Freshwater Mussels to coordinate a nationwide conservation program (National Native Mussel Conservation Committee, 1998). This document elaborates on the genetic concerns expressed in the national strategy.

Propagation and culture of endangered mussel species typically

is recommended in recovery plans (e.g., US Fish and Wildlife Service (USFWS) 2004), to augment population sizes and to re-introduce species to sites within their historical ranges. A joint policy concerning controlled propagation was adopted by USFWS and the National Marine Fisheries Service (NMFS) to provide guidance and consistency for implementation of species recovery activities involving captive propagation (USFWS and NMFS, 2000). This policy recognizes controlled propagation as a useful tool for establishing new, self-sustaining populations; for supplementing or enhancing wild populations; and for holding offspring of listed species for part of their development if suitable natural conditions do not exist (USFWS and NMFS, 2000). Over the last 10 y, propagation technology has been developed at the Freshwater Mollusk Conservation Center at Virginia Polytechnic Institute and State University (Virginia Tech) and at other facilities in the United States to produce endangered juvenile mussels for this purpose (Neves 2004). Currently, 15 federal and state facilities propagate freshwater mussels in the Southeast and Midwest: Alabama Department of Conservation and Natural Resources, Kentucky Department of Fish and Wildlife Resources, Mammoth Cave National Park (Kentucky), University of Minnesota, North Carolina State University, Ohio State University, Southeast Aquarium Research Institute (Georgia), Southwest Missouri State University (SWMSU), Tennessee Tech University, USFWS Genoa National Fish Hatchery (Wisconsin), USFWS Mammoth Springs National Fish Hatchery (Arkansas), USFWS Warm Springs National Fish Hatchery (Georgia), USFWS White Sulphur Springs National Fish Hatchery (West Virginia), Virginia Department of Game and Inland Fisheries, and Virginia Tech University. These facilities have conducted critical life history studies on freshwater mussels (e.g., Jones & Neves 2002; Neves 2004) and, during the past several years, have released over 1 million juveniles of more than a dozen endangered species into rivers throughout the eastern United States. Survival of laboratory-reared juveniles 1–3 y of age after

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release already has been documented. For example, researchers at SWMSU produced thousands of juvenile Neosho mucket, *Lampsilis rafinesqueana* (Frierson, 1927), and reintroduced them in 2000 into historical habitat in the Fall and Verdigris rivers, Kansas. Biologists recovered 28 juveniles of this species at release sites in 2002 (C. Barnhart, SWMU, pers. comm. 2003). The endangered Higgin's-eye pearlymussel, *Lampsilis higginsii* (L. Lea 1857), and endangered oyster mussel, *Epioblasma capsaeformis* (L. Lea, 1834), have been propagated, outplanted, and recovered at release sites in the upper Mississippi River, Wisconsin, and Clinch River, Tennessee, respectively (R. Gordon, USFWS, Genoa National Fish Hatchery, pers. comm. 2002, Jones & Neves, unpubl. data, 2004). Therefore, propagation of mussels offers state and federal hatcheries an opportunity to expand their mission and assume an important role in conservation of biological diversity in the United States.

Federal and state biologists are optimistic about using propagation technology as a recovery tool for endangered mussels, and as mitigation for mussel populations killed by toxic spills or other anthropogenic impacts. However, as these programs mature and become more successful, the genetic implications of releasing captive-reared progeny to natal or other rivers must be considered. Propagation programs will be challenged to increase population sizes, whereas simultaneously trying to avoid negative consequences of altering the genetic resources of populations (Miller & Kapuscinski 2003). Because little is known about conservation genetics of mussels, researchers and natural resource managers will have to apply the science developed by professionals involved in conservation genetics of fishes, marine bivalves, and other organisms (Lannan 1980a, Lannan 1980b, Meffe 1986, Gaffney et al. 1993, Waples 1999, Hallerman 2003).

In this paper, we discuss application of the principles of conservation genetics to protect genetic resources of mussel populations. Our intent is to identify and justify basic, practical, genetic guidelines for their captive propagation. Readers should be aware that the current state-of-knowledge concerning mussel propagation technology is still in its infancy. Hence, some of the population genetic concerns presented are based upon theoretical principles. Key biological information often is lacking for mussels (e.g., population genetic structure, degree and distribution of adaptive genetic variation, numbers of juveniles needed to demographically boost and effectively restore populations, robust estimates of juvenile mortality in the laboratory and field, and effective and minimal viable population sizes). Therefore, questions concerning effects of artificial propagation technology on variation of adaptive genetic traits (e.g., life history traits) are yet unanswered. Propagation programs must take an adaptive approach to management of mussel resources, one that readily learns from results and applies best available science to conservation goals. Ten guidelines are discussed in this paper and are primarily aimed at avoiding genetic hazards associated with implementation of hatchery supplementation programs (Table 1). The intent of this paper is to remind mussel culturists of basic genetic guidelines and protocols to help protect genetic resources of propagated mussel species. We anticipate that as propagation technology advances, more sophisticated genetic management guidelines and plans will be needed to advise hatchery managers.

#### *Life History of Freshwater Mussels*

Development of conservation strategies unique to mussels must be grounded in an understanding of their life histories, population

genetic structure and population dynamics. Mussels are suspension-feeders that live most of their lives embedded in the gravel, sand or mud substrates in rivers or lakes. They are generally long-lived animals that exhibit slow to moderate population recruitment rates. Many species commonly live for more than 20 y, with some living more than 150 y (Zingarov et al. 1998). Eggs of female mussels are fertilized internally by sperm released by males into the water and taken in by females during siphoning. The sexes are separate in most species, but some species are hermaphrodites (van der Schalie 1966, 1970). The embryos then develop in the gills of the female until becoming mature parasitic larvae (glochidia). Once the glochidia are mature, the female releases them into the water, where they must attach and encyst on the gills, fins or epidermis of a suitable host fish for metamorphosis to the juvenile stage. Glochidia of most mussel species require specific fish hosts to transform into juveniles and disperse into new habitats. To maximize attachment of glochidia to host fish, some mussel species produce glochidia in packets (conglutinates) or have mantle-tissue modified into the shape of lures that closely resemble prey items (Fig. 1). Female mantle tissue and conglutinates can mimic insect larvae and pupae, leeches, flatworms, and even other fish, all of which seem to attract host fish closer for possible infestation by glochidia (Parmalee & Bogan 1998). Metamorphosis typically requires 2–3 wk, depending on seasonal water temperatures. Once this parasitic transformation is complete, juveniles excyst and drop from the fish host to begin their lives on the bottom of a river or lake. The juvenile must settle into suitable substrate to have a high likelihood of survival.

Basic life history data, estimates of population size, and assessments of population genetic structure are lacking or sparse for many endangered mussel species. However, this information is critical for making sound management decisions during captive propagation of species. Thus, it is imperative that natural resource managers and administrators recognize that meeting many of the guidelines discussed in this paper will require that studies be conducted to assess population size, population genetic structure and life history parameters prior to implementation of propagation activities for some species, especially when multiple populations of a species exist and augmentation is an intended recovery strategy. In the remainder of the paper, we discuss the genetic issues that should be of concern to mussel culturists, each followed by its recommended guideline.

#### *Addressing Causes of Decline and Extinction*

The decline of mussel species throughout North America in the 20th century is attributed to degradation of habitat from various factors, including channelization, damming, mining, pollution, residential development, silting of rivers, and more recently, competition with the exotic zebra mussel *Dreissena polymorpha* (Pallas 1771). Dams change the flow, temperature and dissolved oxygen regimes of free-flowing rivers, such that the reproductive cycle of freshwater mussels is disrupted; gametogenesis is inhibited and fish hosts that prefer shallow, free-flowing river habitat are extirpated from impounded reaches. Thus, dams prevent or inhibit dispersal of mussels, limiting their ability to recolonize historic habitats and sustain natal ranges. Pollution and siltation of rivers degrades benthic habitats and interferes with osmoregulation, feeding and survival of adults and juveniles. Zebra mussels attach to the shells of native mussels and directly interfere with feeding, respiration and reproduction, causing a decline in physiological condi-

TABLE 1.

Summary and justification for the 10 genetic guidelines recommended in this paper for captive propagation of freshwater mussels.

	Summary	Justification
Guideline 1:	Threats to population persistence should be identified and, when feasible, corrected prior to implementing captive propagation for a species.	Increases availability of suitable habitat for population restoration
Guideline 2:	Each mussel species targeted for recovery using propagation technology should have a recovery plan that defines: (1) necessity of genetic characterization of remaining populations; (2) number of populations to be augmented or reintroduced to effectively recover the species; (3) appropriate locations for release of juvenile mussels; (4) number of juveniles to be released per year at a site; (5) number of gravid females to be collected per year for broodstock and (6) field and laboratory protocols to minimize genetic risks incurred by recovery activities.	Promotes implementation of hatchery activities using approved plans designed to protect genetic resources of populations
Guideline 3:	Collection of gravid female mussels for an augmentation ideally should come from the natal river, or from the closest genetically similar viable population, and that for restoring species into historical river habitat from the closest adjacent river system.	Maintains within- and among-population genetic variation
Guideline 4:	Establish an appropriate number of gravid females to be collected each year for propagation from a small population, as well as protocols to monitor survival and recruitment of artificially propagated juveniles.	Minimizes over-collection of broodstock from small populations
Guideline 5:	Maintain the largest possible genetically effective population size ( $N_e$ ) of propagated juvenile mussels by collecting an appropriate number of adult females each year to use as broodstock, and when feasible, rotate broodstock periodically.	Maintains within-population genetic variation
Guideline 6:	To avoid declines in population fitness due to outbreeding depression, populations that qualify as evolutionarily significant units (ESUs), subspecies, or closely related species should not be mixed.	Maintains among-population genetic variation
Guideline 7:	Reduce domestication selection during propagation and culture of juvenile mussels by mimicking natural life history processes, such as fish hosts, diet, temperature regimes, and habitat of a targeted species as closely as possible in the hatchery.	Increases progeny fitness and survival when released to the wild
Guideline 8:	Protocols are needed to prevent mixing of species or other management units through inadvertent exchanges of juveniles on laboratory equipment.	Maintains among population genetic variation
Guideline 9:	Release an appropriate number of juvenile mussels from an appropriate number of parents at release sites to maximize effective population size ( $N_e$ ), and at an early life stage to maximize survival in the wild, and to minimize the effects of domestication selection.	Maintains within population genetic variation and reduces domestication selection
Guideline 10:	Monitoring, evaluation, and database management should be regarded as an integral part of any augmentation or restoration program, followed as appropriate with modification of program goals and operations procedures to promote program effectiveness.	Promotes program effectiveness and adaptive management

tion and eventual death (Haag et al. 1993, Neves 1999). Both habitat degradation and nonindigenous species accelerate native mussel population declines by negatively affecting vital rates, notably reproduction, recruitment, survival and dispersal. Identifying threats to population persistence in species targeted for recovery is an important step in determining the feasibility and necessity of captive propagation. Only when the causes of decline are identified and corrected can conservationists effectively implement augmentations and reintroductions to remedy small population problems (Caughley 1994) and re-establish populations within historical ranges. Hence, propagation programs should be viewed as a re-

covery tool that is integrated within larger ecosystem management programs of habitat protection and restoration. Propagation of endangered mussel species is a supplement rather than a substitute for addressing factors responsible for population declines.

*Guideline 1:* Threats to population persistence should be identified and, when feasible, corrected prior to implementing captive propagation for a species.

#### *Propagation and Recovery Goals*

Because species conservation units are identified by genetic studies, the focus of recovery efforts for some species will shift to



**Figure 1.** (A & B) Phenotypically variable mantle displays of the wavy-rayed lampmussel *Lampsilis fasciola* mimic prey of predatory fish hosts, (C) Conglutinates of kidneyshell *Ptychobranchius fasciolaris* mimic the larval stage of blackflies (Simuliidae) to attract darter hosts, and (D) Conglutinates of fluted kidneyshell *Ptychobranchius subtentum* mimic the pupal stage of blackflies (length of conglutinates is 3–5 mm). Photographs by Jess Jones.

implementation of a captive propagation program. Hatcheries will be used to produce and release sufficient numbers of juvenile mussels of suitable physiological and genetic quality to alleviate the immediate threat of extinction for an endangered mussel species, and to demographically boost a population to the point where it is self-sustaining. Species should be prioritized for recovery based on their risk of extinction using analytic tools such as population viability analysis (PVA) (Beissinger & McCullough 2002). Accomplishing these goals will require restoration, augmentation and protection of viable populations of targeted species and their habitats, and continued research into their life history and population dynamics. *Restoration* is the re-establishment of populations into historical habitats from which the species has been extirpated, whereas *augmentation* is the rehabilitation of demographically depressed populations with translocated adults or hatchery-reared progeny. To achieve these goals, propagation programs will need to adopt straightforward guidelines to help protect genetic resources of species prior to initiating captive propagation activities.

Criteria for down-listing endangered species to threatened and ultimately to recovered status are stated in federal recovery plans (e.g., USFWS 1984, 2004) and are useful for developing propagation goals. These plans provide basic biological information pertinent to the recovery of a species. In addition to biological requirements, recovery plans typically require the existence of 3–6

(e.g., 6 for *Epioblasma capsaeformis*), and sometimes more, distinct viable populations of a species for down-listing from endangered to threatened (USFWS, 2004). Plans define a viable population as a wild, naturally reproducing population that is large enough to maintain sufficient genetic variation to enable the species to adapt and respond to natural habitat changes without further intervention (USFWS 2004). Populations are considered distinct when they are sufficiently separated such that a single mortality event would not eliminate or reduce more than one population.

**Guideline 2:** Each mussel species targeted for recovery using propagation technology should have a recovery plan that defines: (1) necessity of genetic characterization of remaining populations; (2) number of populations to be augmented or reintroduced to effectively recover the species; (3) appropriate locations for release of juvenile mussels; (4) number of juveniles to be released per year at each site; (5) number of gravid females to be collected per year for broodstock and (6) field and laboratory protocols to minimize genetic risks incurred by recovery activities.

#### *Genetic Hazards and Risks*

Hatchery and field activities associated with captive propagation programs pose genetic hazards for a targeted population. A hazard is an adverse genetic consequence of hatchery activities on



a population, and a risk is the probability that a hazard will occur (Busack & Currans 1995). Four types of genetic hazards have been identified: (1) extinction; (2) loss of within-population genetic variation; (3) loss of between-population genetic variation and (4) domestication selection (Busack & Currans 1995). The risk is generally low for causing the extinction of a species (Type 1 Hazard) by recovery activities of a hatchery program; however, the over-collection of broodstock warrants further consideration, to be discussed in the next section. The loss of within-population genetic variation (Type 2 Hazard) is generally caused by propagation of progeny from a limited number of parental broodstock. Loss of within-population genetic variation is accelerated when only a few adults are used as broodstock to produce progeny for release back into the natal population or when there is high variance of reproductive success among breeders (Hallerman 2003). The loss of between-population variation (Type 3 Hazard) is caused when genetic distinctiveness is reduced or lost because of mixing populations that otherwise would not interbreed naturally through migration. Because scientists are still uncertain of the effects of losing genetic variation on mussel population fitness, cognizant hatchery personnel should attempt to minimize human-caused losses of genetic variation (Hard 1995, Waples 1999). Domestication selection (Type 4 Hazard) is the consequence of any change in the selection regimen experienced by a cultured population, relative to what it would have experienced in the wild (Waples 1999). Hatcheries can alter selection regimes in several ways (discussed in detail later). Therefore, personnel involved with the design and implementation of hatchery supplementation programs need to recognize genetic hazards and understand how to avoid or minimize risks associated with propagation activities of targeted species, as discussed in the sections that follow.

#### *Selection of Broodstock Source Populations*

Gravid female mussels typically are collected directly from their natal river for use as hatchery broodstock. Populations in close proximity to one another within a river basin are typically best suited for use as broodstock to restore or augment adjacent populations with propagated juveniles. Hence when possible, collection of gravid females for augmenting a population should come from the natal river. Restoration of a species into an historical stream of occurrence should use broodstock from the closest adjacent watershed based on stream distance and with the most similar genetic and ecological characteristics. Source populations should be similar to the recipient population based on: (1) genetic lineage; (2) life history patterns; (3) ecology of the originating environment and (4) physiographic division (Miller & Kapuscinski 2003). In regards to the last factor, the close proximity of populations does not preclude the need for genetic analysis, especially for mussel species that have limited dispersal capabilities and occur in smaller headwater streams, such as some *Epioblasma* and *Pleurobema* spp. that use darters and minnows as hosts. Fine-scale geographic patterns of genetic variation may exist for these species. In such cases, the desire to preserve native population genetic structure (to avoid Type 3 Hazard) must be carefully balanced with the need to augment the population with progeny from a population in another stream. Further, viable populations of many endangered species are few, and some species are reduced to a single population. In these cases, the need for among-population genetic analysis will be limited or not necessary, and selection of source populations for translocation or captive propagation generally can be based on geography alone or criteria to prevent extinction.

*Guideline 3:* Collection of gravid female mussels for an augmentation ideally should come from the natal river, or from the closest genetically similar viable population, for restoring species into an historical river, from the closest adjacent river system.

Collection of an excessive number of adult female mussels for broodstock from a population can effectively "mine" natural populations by removing reproductive individuals from their source population and potentially contribute to decline (Type 1 Hazard, Miller & Kapuscinski 2003). This can happen when the survival of hatchery-reared progeny is less than survival of those produced naturally. For critically endangered species comprised of a single small population, it may be necessary to establish a maximum number of females to be collected each year for use as broodstock. This practice can help prevent over-collection of gravid females from a population and allow for some level of annual *in situ* reproduction to occur. For example, the population of endangered tan riffleshell *Epioblasma florentina walkeri* (Wilson & Clark 1914) in the Clinch River watershed occurs only in a 1,200 m reach of a tributary stream. The population size has been estimated at  $n = 2,000$  (Rogers et al. 2001). However, based on field observations of the number of gravid females releasing glochidia each year in the spring (J. Jones, unpublished data), the actual number of breeding females is much smaller. In such cases, establishing an appropriate number of gravid females to be collected each year for broodstock from a small population is a prudent measure to ensure continuation of annual *in situ* population reproduction. In addition, it is important to monitor the success of propagation efforts, to determine whether recruitment of hatchery-reared juveniles exceeds that of naturally produced juveniles, and that artificial propagation truly contributes to an increase of the targeted population.

*Guideline 4:* Establish an appropriate number of gravid females to be collected each year for propagation from a small population as well as protocols to monitor survival and recruitment of artificially propagated juveniles.

#### *Maintaining Genetic Resources of Cultured Mussel Species*

The American conservationist Aldo Leopold (1949) once stated that the art of successful tinkering requires that we first save all of the parts. Leopold's advice certainly is applicable to conservation of genetic resources of propagated species; however, heeding this advice will require that culturists have detailed knowledge of the genetic composition of populations and an understanding of the effective population size ( $N_e$ ) needed to maintain appropriate levels of genetic diversity. Genetic studies will be needed to elucidate the genetic structure of populations, especially to determine the presence and proportions of rare alleles in populations. Once this information is available, an appropriate broodstock effective population size ( $N_e$ ) can be determined to maintain genetic variation.

The effective population size ( $N_e$ ) is defined as: the size of an *idealized population* that would lose genetic diversity at the same rate as the actual population under consideration (Kimura 1983). An idealized population assumes: (1) no migration; (2) distinct, nonoverlapping generations; (3) number of breeding adults is the same in all generations and (4) all individuals are potential breeders (Kimura 1983). Furthermore, it is assumed that all individuals in an idealized population randomly mate, and the population is closed in all succeeding generations; other simplifying conditions exist as well for an idealized population. Obviously, riverine populations of mussels do not meet these conditions, but the behavior

of how genes are transmitted from generation to generation in an idealized population provides useful theoretical predictions about how real populations can lose genetic diversity. For example, if a real population loses genetic diversity at the same rate as an idealized population of 100, then the  $N_e$  of the real population is 100, even if it contains 1,000 individuals (Frankham et al. 2002). For many wild populations, the estimated ratio of effective population size to census population size ( $N_e/N_c$ ) is approximately 10% (Frankham et al. 2002). Hence, the actual number of breeding adults in a natural or captive population contributing their offspring to the next generation is considerably less than the census size of a wild or broodstock population.

Populations of imperiled mussel species often are small and susceptible to loss of genetic variation through ecological, demographic and anthropogenic factors, to include artificial propagation. Furthermore, once these populations become small, genetic variation typically is further eroded by nonselective forces, such as inbreeding and genetic drift. Random genetic drift occurs at a rate inversely proportional to the genetically effective population size ( $N_e$ ) (Kimura & Crow 1963). Importantly, loss of within-population genetic variation (Type 2 Hazard) can result in a reduced capacity of populations to adapt to changing environments, which is manifested as a decrease in fitness of individuals within a population (Meffe 1986). Because management for a large  $N_e$  is necessary to avoid inbreeding and loss of genetic variation, what, then, are guidelines that mussel culturists and biologists can follow to accomplish these goals? Popular management guidelines—such as the “50/500 rule,” which recommends an  $N_e$  of 50 to prevent inbreeding depression and 500 to prevent long-term erosion of genetic variability by genetic drift (Frankel & Soule 1981)—are helpful but often impractical for critically endangered mussel species. Therefore, a long-term strategy is needed to increase  $N_e$  over many year-classes, especially for small populations. In addition, because little is known of mussel reproductive biology (i.e., fertilization success rates) equal sex ratios may have to be assumed. For example, if 10 gravid females are collected as broodstock, it might be assumed that each female was fertilized by one male, and therefore,  $N_e = 20$ . However, it is likely that  $N_e$  is much lower in natural populations of some species because of hermaphroditic reproduction and low fertilization success between males and females. A target sample of 20–25 randomly collected animals can contain ~98% of the expected heterozygosity of a wild population (Lacy 1994), and could be achieved for even small populations over 1–5 y. Accordingly, multiple gravid female mussels should be collected annually from various sites to represent a range of river locations, habitats and subpopulations within the source population. Larger ( $n > 5,000$ ) populations of an endangered mussel species are likely to contain considerably higher amounts of genetic variation; therefore, collection of a greater number of gravid females per year is necessary to increase  $N_e$  and genetic diversity of propagated cohorts over time. Other researchers have recommended collecting a minimum of 50–200 individuals to serve as broodstock (Ryman & Stahl 1980, Allendorf & Ryman 1987). Such a strategy helps ensure that any rare alleles (e.g., those at a frequency of <5%) occurring in a population are adequately represented in the broodstock and subsequent progeny. Thus, for larger populations where collection of gravid females can easily be accomplished, it is recommended that >50 individuals be targeted over time to augment or re-establish populations. All females should be tagged prior to their release back to the river or if held in a hatchery as captive broodstock. This will prevent excessive

use and over-representation of the genomes of a limited number of females (see discussion of Ryman & Laikre [1991] effect below). In addition, tagged mussels can be tracked in the field and hatchery for survival and subsequent gravidity. In the future, factorial mating designs (in which males and/or females are mated with multiple members of the other sex) might be used to increase genetically effective population size of hatchery-produced progeny. Thus, with time, we hope to gain the ability to implement direct matings and thereby minimize loss of within-population variation.

*Guideline 5:* Maintain the largest possible genetically effective population size ( $N_e$ ) of propagated juvenile mussels by collecting an appropriate number of adult females each year to use as broodstock and, when feasible, rotate broodstock periodically.

Because the effect of loss of genetic diversity in mussel populations is unknown, management of effective population size and genetic variation for mussel species should be a primary concern to biologists and culturists. However, technical constraints confronting propagation of some endangered mussel species dictate that these genetic concerns will be difficult to accommodate initially. Some species are now sufficiently rare, that obtaining even a few gravid females per year for propagation is difficult (Rogers et al. 2001, Jones et al. 2004). The high fecundity and output of glochidia by individual females provides an opportunity to produce many more juveniles than would have survived in nature; such recovery opportunities should be exploited to alleviate demographic and environmental threats to persistence of small populations. Thus, in the initial stages of recovery for some endangered mussel species, increasing population density to alleviate immediate threats to population persistence will have to be weighed against managing for increasing genetically effective population size and genetic diversity. Clearly, there is a need to balance our capacity to produce and release numerous progeny while trying to maintain genetic diversity of populations (Type 2 and 3 Hazards).

#### *Outbreeding Depression*

Outbreeding depression is a decrease in fitness of progeny upon breakup of coadapted gene complexes resulting from mating of distantly related individuals (Dobzhansky 1937). Although untested in freshwater mussels, outbreeding depression has posed a threat to population viability in some species of marine bivalve mollusks (Lannan 1980a, Lannan 1980b, Gaffney et al. 1993, Boudry et al. 2002). We hypothesize that mussel species and populations that have limited dispersal capabilities and that are subject to local environmental selection pressures may have developed coadapted gene complexes for adaptation to such environments, to include local host fish communities. For example, recent research on fish host specificity has demonstrated that glochidia obtained from allopatric mussel populations can exhibit significant among-population variation in transformation success when exposed to local fish host communities (Rogers et al. 2001, Eckert 2003, Jones et al. 2006). Other factors, such as differences in various life history parameters (e.g., spawning seasonality), population demographic parameters, physiological response to water quality (e.g., differences in local geochemistry) and other potentially adaptive traits should be assessed by biologists. Thus, we suspect that some populations of freshwater mussels may be vulnerable to outbreeding depression, and mixing distinct populations may disrupt genetic adaptation to local environmental conditions.

*Guideline 6:* To avoid declines in population fitness caused by outbreeding depression, allopatric populations that qualify as evo-

lutionarily significant units (ESUs), subspecies, or closely related species should not be mixed.

#### *Domestication Selection*

Domestication selection (Type 4 Hazard) causes genetic changes in captive-held populations. In the captive rearing environment, artificial selective forces can replace those of natural selection. Domestication selection occurs because a different set of progeny survive in the hatchery than would have survived in the wild. Genetic changes can affect morphological, physiological, or behavioral traits and lead to decreased performance and survival of captive-reared progeny in natural environments. Because mussel propagation is still in its infancy, domestication selection has not been documented in the rearing of a mussel species; however, it has in the rearing of fishes (Miller & Kapuscinski 2003) and marine bivalves in hatcheries (see annotated bibliography by Moore & Seeb 2001). For example, many salmon hatcheries producing fish to augment wild populations are careful to collect breeders from different time-periods through the spawning run of a particular stock. This field-collection practice allows genetic representation of breeders that collectively spawn from early to late in the run. Similar practices may be necessary for some species of mussels to prevent artificial selection. For example, females of the endangered oyster mussel *Epioblasma capsaeformis* in the Clinch River, Tennessee, typically begin displaying their mantle-pad lure and releasing glochidia to host fish in April and continue into June (Jones et al. 2005). Some individual females display early in the spring, whereas others display much later. These differences in the timing of release of glochidia by *E. capsaeformis* may be genetically controlled and suggest that gravid females should be collected at different times throughout the glochidial release period. If, for example, time of glochidial release is under genetic control, then excessive propagation and release of juvenile mussels from females collected in the early spring could shift forward the glochidial release period of a targeted population relative to that of the wild population.

Research is needed to determine how domestication selection could alter the genetics of captive-reared juvenile mussels through stages in the propagation process, including investigation of the following: (1) most appropriate time of year to remove glochidia from the female mussel to maximize maturity of glochidia (Jones et al. 2005); (2) use of marginally-suited host fish for transforming glochidia to the juvenile stage; (3) appropriate diet, optimum substratum, exposure to disease and rearing temperatures and (4) length of culture period in captivity before release to the wild. To minimize domestication selection, we must clearly understand natural regimes and requirements for fish host usage, grow-out temperature, substrates, growth rates, light regimen, diet and size of juveniles at release relative to naturally-produced juveniles, thereby mimicking the ecology and habitat of a species as closely as possible throughout the propagation process (Maynard et al. 1995, Flagg & Nash 1999).

*Guideline 7:* Reduce domestication selection during propagation and culture of juvenile mussels by mimicking natural life history processes, such as fish hosts, diet, temperature regimes, and habitat of a targeted species as closely as possible in the hatchery.

#### *Laboratory Protocols to Prevent Mixing of Mussel Species*

The establishment of laboratory protocols to prevent the inadvertent mixing of species or other management units is important

to protect the integrity of genetic resources. Most propagation facilities rearing juvenile mussels for augmentation or restoration are cultivating multiple species and populations from different drainages. For example, at the Freshwater Mollusk Conservation Center at Virginia Tech University, juveniles of 6–9 endangered mussel species are produced each year, representing species from several major river drainages. In these situations, separate tank systems are required for holding host fish and for grow-out of juveniles from different drainages. Because juvenile mussels are small (~200–1,000  $\mu\text{m}$ ) for the first 60 days of life and can easily attach to laboratory equipment used for handling juveniles, such as sieves, siphons and Petri dishes, these items also should be kept separate for each lot and disinfected regularly. All hatchery personnel should be trained in field and laboratory protocols to reduce the risk of unintentional mixing of cultured populations.

*Guideline 8:* Protocols to prevent mixing of species or other management units through inadvertent exchanges of juveniles on laboratory equipment are needed to protect genetic resources of freshwater mussel populations.

#### *Release of Propagated Juveniles*

A suite of factors should be considered before juvenile mussels are released to the wild. Such planning is especially important for critically endangered populations with small effective population sizes ( $N_e$ ). Small populations (e.g.,  $n = 500$ –1,000 and  $N_e < 50$ –100) warrant special attention if they serve as a source for augmentation or reintroduction. First, production and release of thousands of juveniles from a small number of adult females into a small ( $n < 1,000$ ) recipient population can significantly decrease  $N_e$ , because of unequal contributions of progeny from only a few progenitors (Ryman & Laikre 1991). Therefore, a target number of offspring should be established for release into a small population prior to augmentation. Excess progeny could be released at adjacent shoals or at other acceptable sites. Second, selection of suitable release sites should be based on at least the following criteria: (1) biological requirements of the species, such as presence of fish hosts; (2) habitat quality and (3) thorough assessment of localized and upstream threats to release sites. Third, juveniles should be released at the earliest life-stage possible that will maximize survival in the wild. There is a trade-off between how long juveniles are reared in the hatchery, to increase survival rate relative to juveniles reared naturally and continued exposure to the hatchery environment and the associated extent of domestication selection (Miller & Kapuscinski 2003). Exposure to natural environmental patterns and selective forces at an early life stage may prove most beneficial to ensure fitness in the wild of hatchery-reared juveniles. Fourth, juveniles should be released under moderate-to-low flow conditions to allow settlement at the selected site on the river bottom, and at the appropriate time of year (spring-summer). Fifth, release methods and sites should be selected to increase the range and connectivity of localized demes and populations. For example, juveniles could be released at suitable sites between known locations of upstream and downstream demes. Sixth, as propagation technology improves and juveniles are grown to larger sizes, juveniles should be marked with a tag or chemical stain to facilitate monitoring efforts (see Eads & Layzer 2002).

The possibility of releasing host fish infested with glochidia would allow natural dispersal and colonization of habitats otherwise excluded by only releasing hatchery-reared juveniles, spread risk of mortality at localized stream reaches, and may minimize

future inbreeding. However, this practice risks loss of juveniles after settlement into unfavorable areas, and makes monitoring of survival success difficult. Under some circumstances, such as in small streams, this strategy may be more effective than site specific releases of cultured juveniles.

**Guideline 9:** Release an appropriate number of juvenile mussels from an appropriate number of parents at release sites to maximize effective population size ( $N_e$ ), and at an early life stage to maximize survival in the wild, and to minimize the effects of domestication selection.

#### Monitoring and Adaptive Management

Captive propagation of mussels is a new recovery option, and is as much an art as a well-established science at this time. Success must be measured not in terms of how many juveniles are outplanted, but rather in terms of how many juveniles recruited into or established a spawning population. Furthermore, data on (1) number of gravid females used to produce juveniles; (2) locations where females were collected; (3) number of juveniles released per site and river location; (4) juvenile characteristics (e.g., age, size and condition) and (5) river conditions at the time of release, should be recorded and submitted to the responsible natural resource agency. Standard data sheets should be prepared and used for all releases. It is critical that protocols to monitor survival and recruitment of artificially propagated juveniles are established and implemented, and project data are collected in an appropriate agency database. Ultimately, success will be measured in terms of the establishment of self-sustaining populations. Hence, monitoring should be regarded as an integral part of any captive propagation and release program.

Because of the many unknowns in mussel biology and uncertainties in long-term effects, hatchery programs may be experimental in nature, but should be integrated into an adaptive management program, with careful attention to monitoring and re-evaluation of goals and protocols. Under the adaptive management paradigm, results of monitoring are used, as appropriate, to modify management goals and operations procedures so that, over time, learning occurs and the overall program becomes more effective

(Holling 1978). Adaptive management has proven useful for management of Pacific salmonids (Hilborn & Winton 1993, Walters et al. 1993), and we acknowledge that it is essential for captive propagation and outplanting of imperiled mollusks.

**Guideline 10:** Monitoring, evaluation, and database management should be regarded as an integral part of any augmentation or restoration program, followed as appropriate with modification of program goals and operations procedures to promote program effectiveness.

#### Concluding Remarks

We advocate application of the principles of conservation genetics to species recovery efforts for freshwater mussels. However, these principles should be recognized as guidelines, and not as goals per se (Neves et al. 1997). Propagation technology and techniques will continue to develop as a recovery tool for a greater suite of species, and to hopefully prevent further extirpations and extinctions. Propagation may effectively alleviate problems associated with small populations, and has the potential to re-establish populations extirpated by known and ameliorated causes. Although propagation offers a wealth of benefits for conservation and restoration, managers of propagation facilities must recognize how each stage in the propagation process can affect the genetic integrity of mussel populations targeted for recovery. A conservation program of sound aquaculture practices, knowledge of the faunal group and application of conservation genetic principles will provide the tools needed to recover and restore species now threatened with extinction.

#### ACKNOWLEDGMENTS

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## ACOUSTIC DETERRENTS DO NOT REDUCE BLACK DRUM PREDATION ON OYSTERS

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**ABSTRACT** We studied the effectiveness of acoustic deterrents in limiting predation on Louisiana oyster leases by black drum *Pogonias cromis*. Three acoustic deterrents were used: (1) natural or synthesized alarm sounds; (2) synthesized, low frequency sound played over short time intervals and (3) mechanically produced sound. Fish locations were monitored in all approaches and feeding rates on oysters were recorded in approaches 1 and 3. Experiments in salt-water ponds indicated that alarm calls from males, or synthesized tapes (approach 1) did not depress fish feeding rates or cause fish to avoid transducers, in comparison with controls. Lower frequencies (<20 Hz, approach 2) displaced fish 8 m further from transducers (about 20% of the distance possible) relative to controls. Finally, a solar-powered hammer (approach 3) was designed as a more logistically feasible deterrent. Fish were weakly attracted (although control and experimental locations differed by only 5%). Because acoustic deterrents were either ineffective at displacing fish or lowering feeding rates on oysters, or required considerable electrical power to displace fish, we conclude that they are not practical to control losses of oysters on leases to black drum.

**KEY WORDS:** black drum, sound deterrents, oysters

### INTRODUCTION

The black drum, *Pogonias cromis* (Linnaeus, 1766), is a significant predator of oysters in the northern Gulf of Mexico (Cave 1978, Sutter et al. 1986, Dugas 1986). Mortality rates are low in intertidal oyster beds because of aerial exposure and the aggregated growth form, but commercial, subtidal oyster leases often experience high mortality. Oyster mortality is especially high after small, individual “seed” oysters are bedded in the fall, or when black drum return to estuarine waters in the spring after breeding in coastal passes. In a survey of lease holders in Louisiana (LADWF 2000), 55% reported black drum caused losses to production. With powerful pharyngeal teeth, black drum crush and eat 30 oysters per night, and black drum >70 cm in length can feed on oysters 75 mm in length (Cave 1978). Our earlier work indicated mortality rates as high as 90% on experimental leases, and that scent deterrents (e.g., from dead con-specifics) were not effective (Brown et al., 2003).

Some evidence suggested that acoustic deterrents might be effective. Black drum are vocal (as are other Sciaenid fishes) and emit low frequency “drumming” sounds, which may attract females or act as alarm calls. These sounds can be heard by humans both in air and water and are produced by muscular contractions against an inflated swim bladder that acts as a resonator (Smith 1905). Black drum detect frequencies ranging from <100–800 Hz and are most sensitive to frequencies <500 Hz (Rameharitar & Popper 2004).

Acoustic cues can also alter fish distributions and feeding rates in other fish. Haymes and Patrick (1986) excluded alewives from water intakes using low-frequency sounds. Ross and Dunning (1996) used 190 dB high frequency (122–128 kHz) broad-based sound, and reduced alewife abundance by 51% at power plant intakes in Lake Ontario. Knudsen et al. (1994) found that salmon (whose hearing range extends from 5–150 Hz), avoid low frequency infra-sound (10 Hz) but not high frequency sounds (150 Hz), and argued that low frequency sounds mimic the swimming

of predators. Maes et al. (2004) used multiple sounds in a frequency range of 20–600 Hz to decrease fish abundance at an estuarine power plant by 60%.

Our research objective was to determine if acoustic deterrents either reduced the feeding rates of black drum on seeded oysters, or caused them to avoid oyster leases. Three general types of acoustic deterrents were used: (1) natural or synthesized alarm sounds; (2) synthesized low frequency sound played over short time intervals and (3) mechanically produced sound. Fish locations were monitored in all approaches and feeding rates on oysters were recorded in approaches 1 and 3.

### MATERIALS AND METHODS

Experiments were conducted in four 0.1 ha saltwater ponds at the Lyle St. Amant Marine Laboratory on Grand Terre Island (29.3°N 89.99°W) operated by the Louisiana Department of Wildlife and Fisheries. Ponds contained pumped-in Barataria Bay sea water (over the experimental interval, mean temperature was 23.4 ± 0.6 °C [±SE], salinity 20.6 ± 0.4 PSU, and dissolved oxygen 8.3 ± 0.4 mg/L) and had removable stand pipes to aid in draining ponds to recapture fish. However, pumps were not run during experiments to eliminate masking of experimental sounds. The ponds were large enough (25 m × 40 m × 1 m deep) so that sound would attenuate with distance (see later in discussion) and reflections would be minimized, allowing drum to find areas with low sound. Although low frequency sounds attenuate rapidly in shallow water over soft sediments (Rogers & Cox 1988), oyster leases are typically seeded under such conditions, and the ponds thus accurately model similar conditions on oyster leases.

In March 2001, using contiguous 30,000 L concrete raceways at the laboratory, we played back tapes with pure sinusoidal wave forms at various frequencies and observed individual black drum. Frequencies from 10 Hz to 100 Hz caused fish to move to the opposite end of the tank. However, there was considerable “cross-talk” (sound transfer among the concrete tanks) along with standing waves that complicated determining the strength of acoustic signals.

Similar studies however indicated a clear gradient in sound

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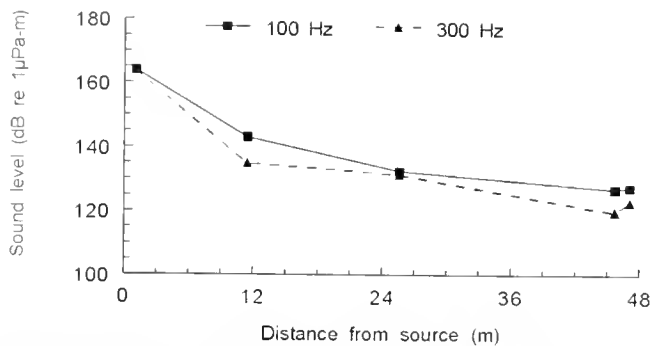


Figure 1. Plot of sound volume detected against distance from the transducer at two frequencies in the experimental ponds.

intensity along the length of the 0.1 ha ponds, similar levels on each side, and little cross talk among ponds (Fig. 1). An Argotech model 210 underwater transducer (positioned at the center of one end of the pond) produced a pure tone of 164 dB  $\mu$ Pa/@ 1 m at a frequency of 100 or 300 Hz. Sound levels were measured with a "Dolphin Ear" DP100-6 hydrophone (frequency response 7 Hz to 22 KHz) and decibel meter at each corner near the transducer (~12.5 m distant), halfway down each side (~23.5 m), and at the center (~40 m) and each corner (~42 m) of the opposite end. At 100 Hz, transmission loss was approximately 90% at 12.5 m, 97% at 23.5 m, and 99% at 40–42 m. At 300 Hz, loss was 96.4% at 12.5 m, 95% at 23.5 m, and 99.2% at 40–42 m. Little variation in signal strength occurred across the pond at any distance, and signals were not detected in adjacent ponds, indicating no cross talk.

For approach 1, experiments were conducted from January to April 2002. Two Argotech model 210 underwater single-neoprene membrane transducers, at the center of one end of each experimental pond, were powered by a 200 W Peavy CS800X amplifier, connected to a cassette recorder. Two continuous-loop tapes were supplied by Argotech. The first was alarm "drumming" by a male in the raceways, edited to remove splashes, and with bandwidth limited to 4 KHz (frequency peaks were at 37, 70, 146, 210 and 300 Hz, Fig. 2). The second was a synthesized recording predicted to produce avoidance based on previous work by Argotech (Fig. 3, with peaks at 20, 40, 60 and 267 Hz).

Transducers were switched weekly, so treatment ponds were controls and the previous controls were treatments, to minimize pond-specific differences in fish feeding rates or habituation. After four weeks, the sound was changed to the synthesized tape (and all fish replaced) and another four, weeklong experiments were run in the same pattern.

Each pond had 5 black drum, with a mean total length of  $82.4 \pm 1.6$  cm (SE) and weight of  $9.9 \pm 0.4$  Kg. Fish were collected by trot line or rod and reel in nearby (<2 Km) Barataria Pass, and were held in raceways. Five fish were used to facilitate tracking (see below). Actual school size has yet to be studied in Black Drum, but we have observed them in both small (~5) and larger groups in Barataria Bay (K. Brown, pers. obs.).

To study feeding, 50 oysters of vulnerable size (50–150 g total wet weight, 5–10 cm shell length, Brown et al. 2003) were placed in each of six Nestler trays (0.67 m<sup>2</sup> bottom area mesh trays with 0.1 m high walls). Three trays were along the short edge of each pond, with the transducer at the center of the trays on one end. No other food was provided. Experiments lasted three days; oysters were replaced daily to determine feeding rates, and trays were then removed for three days to starve fish before the next experiment.

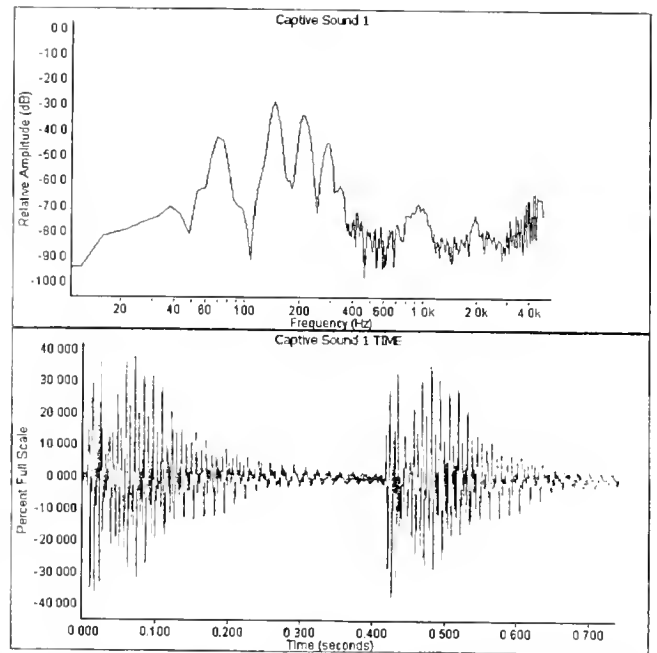


Figure 2. A frequency domain plot (top) and time domain plot (bottom) of an alarm call recorded from a male black drum in the 30,000 L raceways. Frequency peaks are indicated in the text.

Feeding rates were analyzed in a 1-way analysis of covariance, with time since the start of the experiments as a covariate to control for seasonal effects.

To follow movement, fish were tagged with numbered "spaghetti" tags inserted below the soft dorsal fin and attached to individually colored small (~2 × 7 cm) styrofoam floats by 200 pound test, monofilament line. Because the floats were small, and black drum are relatively large fish, they did not impair movement or grouping patterns. Locations (to the nearest 0.5 m) were recorded hourly from 10:00–16:00 on a grid laid out in the ponds, and distances from the transducer digitized. The hourly mean distance of the five fish from the transducer (or the same location in the control ponds) was analyzed in a 1-way repeated measures analysis of variance, contrasting the controls and two recorded sounds. The repeated measure was hour of day.

The second approach involved broadcasting pure tones at frequencies of 10, 15, 20, 25, 30, 40, 50 and 60 Hz in a single pond. The same amplifier powered a single, larger Argotech model 220

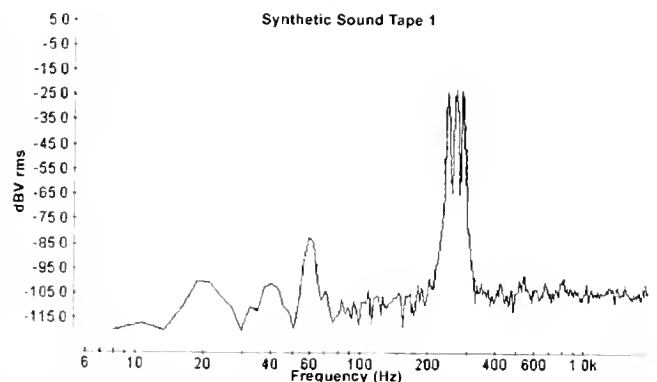


Figure 3. Frequency domain plot of the synthetic tape produced by Argotech and used in approach 1.



twin-neoprene membrane transducer at the center of one end. Although lower-frequency sounds attenuate rapidly in shallow, soft-bottomed environments (see earlier discussion), and we did not explicitly measure attenuation in this experiment, signals carried far enough to displace fish (see results), and were thus apparently audible. Following Maes et al. (2004), we contrasted fish positions during sound-on and sound-off periods. Each experiment had a five-minute interval with sound, and five minutes without. In this experiment, 4 tagged fish were used, and we used similar methods to record the positions of the tagged fish (mean length =  $94.7 \pm 2.3$  cm, mean weight =  $13.0 \pm 0.9$  KG) each minute. The amplifier was then turned off for five minutes before the next frequency was tested. The experiment was repeated the next day, so that 10 replicate observations were recorded for each frequency and its corresponding control period. The locations of the four fish relative to the transducer at each minute interval were analyzed in a 2-way analysis of variance (sound on or off versus frequency). Experiments were conducted from December 6 to 8, 2002.

For the final approach, we collaborated with biological engineers at Louisiana State University's Department of Biological Engineering to produce a mechanical, sound-producing device. The rationale was 2-fold. First, the underwater transducers required considerable power, and were prone to leaks. Second, striking a metal oil drum with a wooden stick near the pond edge caused fish to rapidly move away. The device (Fig. 4) consisted of two 55 gallon, sealed steel drums that provided buoyancy. One drum had an internal electric motor (Fig. 4, inset) activating a spring-loaded hammer. Both the time between impacts and the number of impacts per application were adjusted several times during the experiment by a dial on the top of the apparatus to limit habituation. The motor was powered by a 12-V automotive battery charged by a solar cell suspended above the drums.

The experiment was conducted in one experimental and one control pond. Four tagged fish (mean length =  $86.4 \pm 1.8$  cm, mean weight =  $10.3 \pm 0.5$  KG) were used in this final experiment in each pond. The apparatus could not be easily transferred among ponds, so experimental and control ponds were not reversed. Each experiment lasted two days. On the first evening, six trays filled with 50 oysters were added, and tagged fish locations determined

for six hours the next day. In earlier experiments, hourly locations were a "snapshot", as fish moved constantly. We therefore noted average fish locations for the first 10 minutes of each hour in this experiment. A repeated measures analysis of variance indicated no repeated measures effect ( $P = 0.15$ ), so all 60 observations ( $6 \text{ h} \times 10 \text{ observations}$ ) were used as replicates in a 1-way analysis of covariance. The experiment was repeated five times during April to May 2003. Number of days since the start of the first experiment was the covariate.

## RESULTS

For the initial approach, feeding rates were not depressed by alarm sounds ( $F_{2,59} = 0.7$ ,  $P = 0.52$ ), in comparison with the synthetic sounds or the control (Fig. 5). On average, fish exposed to alarm sounds consumed 49% of the oysters provided daily, in comparison with 67% with synthetic tapes, and 61% in controls. The covariate was significant ( $F_{1,59} = 22.6$ ,  $P < 0.0001$ ) because feeding increased with warmer temperatures or acclimation of fish.

Hour of observation did not affect fish location (Wilk's  $\lambda = 0.83$ ,  $F_{6,46} = 1.5$ ,  $P = 0.19$ ), nor was there a treatment effect ( $F_{2,51} = 0.4$ ,  $P = 0.7$ ). There was an interaction (Wilk's  $\lambda = 0.63$ ,  $F_{12,92} = 2.0$ ,  $P = 0.04$ ), although inspection of the pattern of treatments across hours (Fig. 6) revealed no obvious trends.

In the second approach with lower-frequency sound, frequency was not significant ( $F_{8,152} = 0.7$ ,  $P = 0.69$ ), but there was a difference in location between when the transducer was on and off ( $F_{1,152} = 116.6$ ,  $P < 0.0001$ ), and an interaction between frequency and sound activation ( $F_{8,152} = 5.7$ ,  $P < 0.0001$ ).

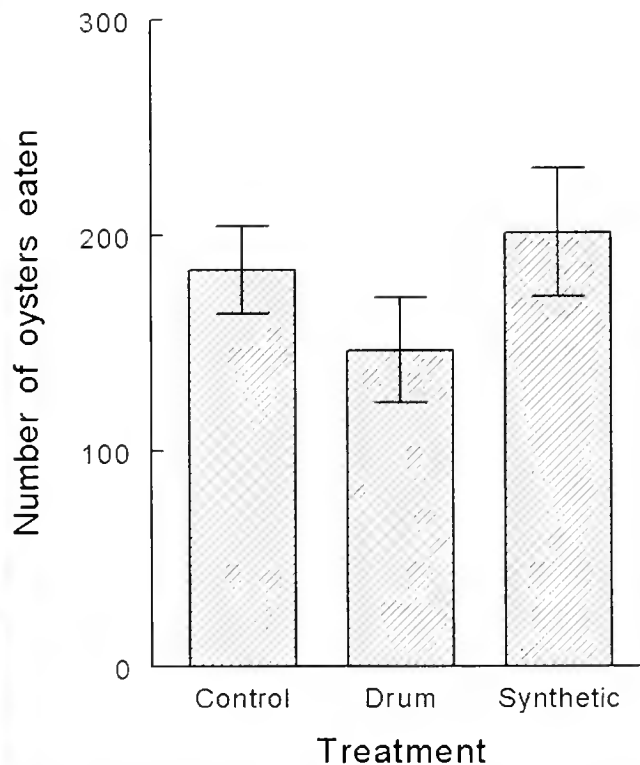


Figure 5. Mean feeding rates on oysters ( $\pm$  SE) from the initial transducer experiment (approach 1). Drum = average for alarm sound, Synthetic = average for synthesized sound, and Control = average without sound.

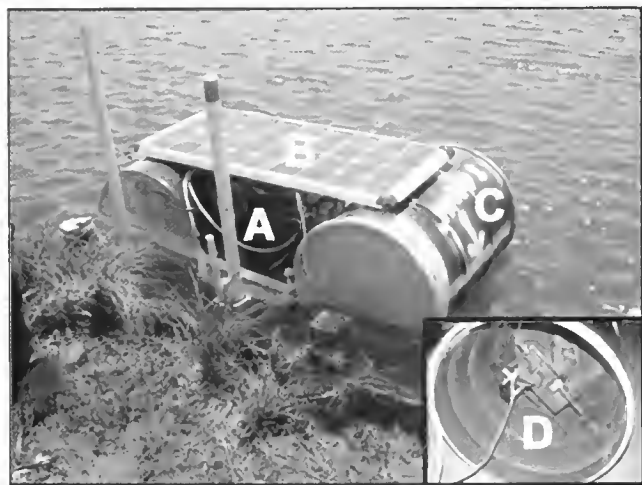


Figure 4. Solar-powered, sound producing mechanism. (A) Automotive battery powering electric motor. (B) Solar cell charging automotive battery. (C) Sealed 55 gallon barrel. (D) Inside barrel showing electric motor and hammer.

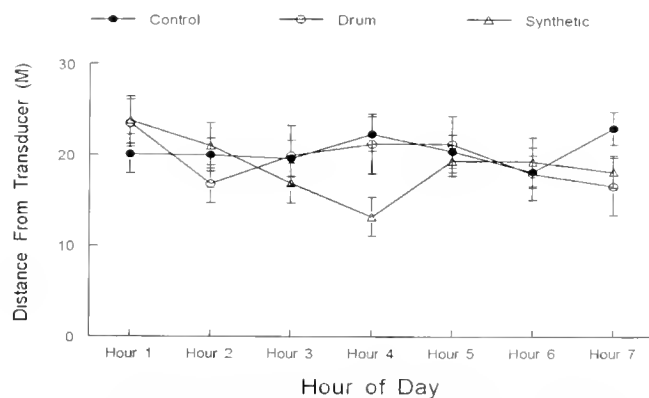


Figure 6. Average locations (distance from transducer,  $\pm$  SE) of fish in the initial transducer experiment, as a function of treatment and hour of the day.

Fish averaged 17 m from the transducer when it was inactive, versus 25.1 m when it was broadcasting. For example, at 15 Hz (Fig. 7), fish moved from less than 10 m to over 33 m away from the transducer within 3 min. and returned to within 15 m after the transducer was switched off. The small standard errors in the figure reflect the close spatial clustering of the fish. The interaction occurred because experimental and control fish locations converged at frequencies  $>20$  Hz (Fig. 8). Comparisons of locations with Tukey *a posteriori* tests indicated fish were further away from transducers at 20 Hz, and closer at 50 Hz, with the distances in all other treatments overlapping. However, eight of the nine highest displacements occurred when the transducer was on, again suggesting the fish were avoiding the lower frequency sounds.

For the final approach with the mechanical device, there was a difference in fish location between the experimental and control ponds ( $F_{1,596} = 8.5$ ,  $P < 0.01$ ). However, the trend was opposite of that predicted, with fish on average 19.2 m away in the control pond, versus 17 m in the experimental pond (Fig. 9). There was also a difference in feeding rates ( $F_{1,18} = 24.6$ ,  $P < 0.0001$ ) with rates in the control pond only 2% of those in the experimental pond (Fig. 9). The covariate did not have a significant effect ( $F_{1,10} = 0.7$ ,  $P = 0.42$ ).

#### DISCUSSION

The first approach suggested that there was little effect of male alarm sounds either in depressing feeding, or in displacing

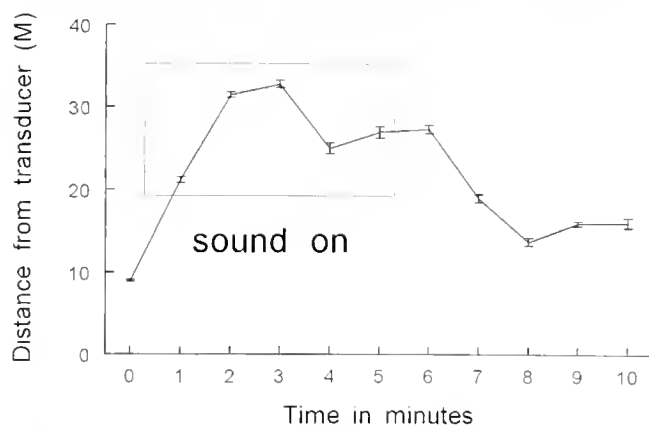


Figure 7. Distance of fish ( $\pm$  SE) from the transducer in the 15 Hz experiment (approach 2). The Box represents the period when the signal was switched on.

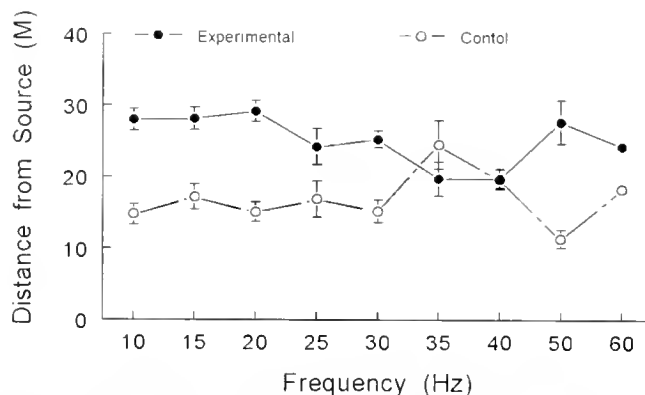


Figure 8. Distance of fish from the transducer ( $\pm$  SE) versus sound frequency. Experimental = sound on, Control = sound off.

black drum from the sound source. Although there were no significant main effects on fish location, variation among the treatments at different times of day caused a significant statistical interaction.

The second approach did indicate displacement of fish from the transducer over the short term at lower frequencies. However, the electrical power necessary to propagate these low frequency sounds underwater makes this approach logistically impractical as a deterrent in field applications (see discussion below). Underwater transducers were also prone to leaks and malfunctions, and had to be replaced on several occasions by Argotech during the experiments.

The results of the first two approaches must be interpreted carefully. Again, low frequency sound attenuates rapidly in shallow, soft-bottomed environments (Rogers & Cox 1988), possibly limiting fish responses. However, the initially measured sound field, although indicating considerable signal loss across the pond, also indicated a sound volume at the opposite end that was still above the threshold of black drum at 100 Hz, about 90 dB re  $1 \mu\text{Pa}$  (Ramcharitar & Popper 2004). Second, fish were evidently able to

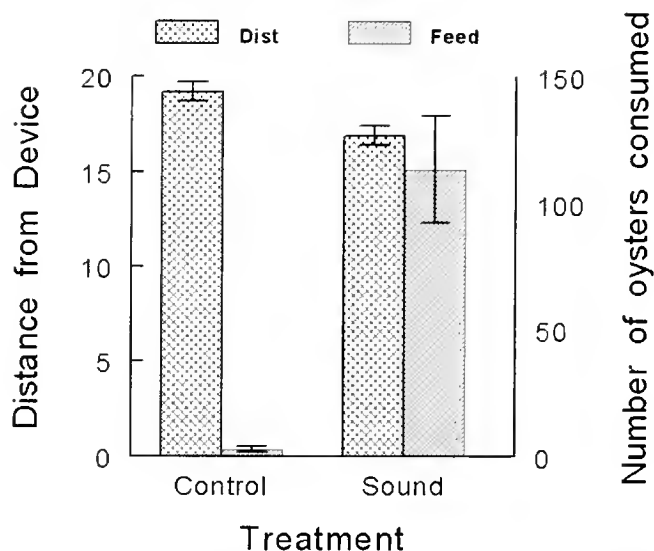


Figure 9. Displacement of fish from the sound-producing device (sound), and feeding rates, in comparison with fish in the control pond (control). Values are means  $\pm$  SE. Dist = distance from device, left axis; Feed = oysters consumed, right axis.

hear the low frequency sounds, because they were displaced. Finally, regardless of the mechanism (whether fish ignored the sounds or could not discern them), the first two approaches were either not effective (approach 1) or practical (approach 2), negating their use in management. We are confident similar results would occur on oyster leases, because the ponds again had similar depth and sediment type.

The last approach, a solar-powered hammer, was more cost effective but weakly attracted fish. However, the average distances differed by only 2 m, only 5% of the distance available. The higher feeding rates in the experimental pond must also be interpreted carefully, because the apparatus could not be moved between ponds. The experimental fish may have been more accustomed to feeding on oysters, similar to differences in feeding between individual groups of fish used in past experiments (Brown et al. 2003).

We conclude that the acoustic deterrents used in approach one and three were not effective for reducing predation by black drum, or could even attract fish. Using sound stimuli at low frequencies

to displace fish (approach 2), although effective, requires more power, and we conclude costs to lease holders would outweigh benefits. Many Louisiana oyster leases are in remote areas, and supplying sufficient electrical power is again problematic. Conducting these lower frequency experiments over longer time intervals might also have revealed habituation in the responses.

#### ACKNOWLEDGMENTS

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## GENETIC DIVERSITY OF THE EUROPEAN OYSTER (*OSTREA EDULIS* L.) IN NOVA SCOTIA: COMPARISON WITH OTHER PARTS OF CANADA, MAINE AND EUROPE AND IMPLICATIONS FOR BROODSTOCK MANAGEMENT

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**ABSTRACT** The European oyster (*Ostrea edulis*) was introduced to the Nova Scotia aquaculture industry 30 years ago using stocks imported from naturalized populations in Maine whose ancestors originated in the Netherlands. This study used 5 microsatellites to assess the level of genetic diversity in several hatchery stocks and naturalized populations from Nova Scotia, New Brunswick, British Columbia and Maine. Some genetic erosion was shown to have occurred in the Maritimes populations, with the largest loss of alleles being experienced by the hatchery stocks. In spite of this loss, genetic diversity and heterozygosity in the Maritimes populations are still relatively high. Relationships within and between the populations and the existence of kin groups within the collections were overall consistent with our knowledge of the historical transfers of oysters between different locations. Furthermore, the established database allowed to assign with good confidence unknown oyster samples to their geographic origin. This would be a useful forensics tool in the case of an illegal transfer from a diseased area.

**KEY WORDS:** *Ostrea edulis*, European oyster, flat oyster, microsatellites, Maine, genetic diversity

### INTRODUCTION

European oysters (*Ostrea edulis*) were introduced to Nova Scotia 30 years ago, principally from naturalized Maine populations whose ancestors originated in the Netherlands in 1949 (Loosanoff 1955, Welsh 1964, Newkirk et al. 1995). They were brought to Nova Scotia to develop oyster aquaculture in the cool, high salinity areas on the Atlantic coast of the province. These waters are generally too cold in the summer to ensure reproductive success of the native American oyster *Crassostrea virginica*, which subsists in only a few small isolated populations in estuaries and tidal lakes (Spares & Dadswell 2001). Growing conditions for *O. edulis* are good in the waters off the Atlantic coast of Nova Scotia but spawning conditions are only marginal in most sites. In addition, the summer season is relatively short, yet good spat growth is essential for ensuring subsequent winter survival. However in a few locations, European oysters have managed to naturalize and have established apparently sustainable populations. Nevertheless, the development of the European oyster industry in Nova Scotia over the past 30 years has relied exclusively on hatchery produced spat that are transferred to grow-out sites at the end of summer/beginning of fall.

There are now separate groups of European oysters in Nova Scotia that may be genetically differentiated to various degrees because of human and environmental influences (e.g., severe winter mortalities in 1990/91 and in 1991/92 or bottlenecks in hatcheries). In addition, the parasite *Bonamia ostreae* is known to be endemic in the European oyster population of Maine but is not present in Nova Scotia. This disease devastated the French oyster industry in 1979 and could have similar devastating consequences if introduced to Nova Scotia, one of the last places in the world where this disease has not been reported. Any importation of new oysters from Maine or other sources is consequently prohibited for this reason.

The preservation and utilization of genetic variability in any broodstock program is a critical and complex issue. Numerous studies have shown that genetic variations can be lost at a rapid rate in hatcheries leading to inbreeding increase and depression (e.g., Beattie et al. 1987, Hedgecock & Sly 1990, Naciri-Graven et al. 2000, see review in Herbinger et al. 2003). In 2001, larvae and spat started to experience massive mortalities in Nova Scotia's hatcheries. Water quality, such as variation in temperature or organic/bacterial load, was suspected to be responsible for the problems, but the possibility of genetic erosion and concomitant increase in inbreeding, coupled with the inability to import new genetic material to Nova Scotia, was also a serious concern. This study was thus concerned with characterizing the present level of genetic variability observed in hatchery and naturalized stocks, the latter being a potential source of *Bonamia*-free broodstock to counteract potential serious losses of diversity in the former. The existing genetic variability and differentiation of various groups of naturalized populations and hatchery stocks of oysters in Nova Scotia was assessed using five microsatellite DNA markers, in comparison with hatchery or endemic *O. edulis* populations in other parts of Canada, Maine and Europe. This study also used DNA marker based pedigree reconstruction to detect the presence of related individuals among any of the groups, as evidence of on-going genetic bottleneck, and it evaluated whether the same microsatellite DNA information could be used to detect illegal importation of oysters from Maine.

### MATERIALS AND METHODS

#### Collection of Populations

Gill or mantle tissue was collected from adult *Ostrea edulis* oysters (>3 y old) using nonlethal sampling techniques (biopsy), and was preserved in 95% ethanol or by freezing at -20°C until DNA was extracted. Samples were taken from 10 sites in 2002 and 2003, which included hatchery stocks from Nova Scotia and British Columbia as well as naturalized populations in Maine (Welsh

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1964), New Brunswick and Nova Scotia (Fig. 1). Figure 1 also summarizes what is known about the various transfer and founding events associated with these populations within Nova Scotia and New Brunswick. As is frequently the case, many of the founding events and transfers are poorly documented. The Maine naturalized populations resulted from the importation of Netherlands oysters in the 1950s (Welsh 1964). In the 1960s, interest in *O. edulis* cultivation developed and several small hatcheries and oyster farms established themselves around the naturalized populations in Maine. The Boothbay and Cundy Harbor populations are older naturalized populations, whereas the Blue Hill Bay population is a more recent "naturalized" population that originated from importations from Boothbay Harbor and from California (Sam Chapman, pers. comm.).

After a small scale unsuccessful importation in the late 1960s to early 1970s from Maine to Nova Scotia, new naturalized oysters from Maine, and a few from North Wales, were imported in 1978 and 1979 to the Dalhousie University hatchery (Halifax, Nova Scotia) (Newkirk, 1986). In 1989 and 1994, respectively, the Dalhousie University and Nova Scotia provincial hatcheries closed and the broodstock was passed on to a total of four small commercial hatcheries, which, in conjunction with several grow-out operations have maintained most of the *O. edulis* cultured populations in Nova Scotia. In one grow-out site (Sambro), a small sustainable natural population managed to establish itself and has survived despite the closure of the grow-out operation. *O. edulis* were also found in Blind Bay, another former grow-out site. However, in this case, no juvenile oysters were recovered, and the few large oysters found appeared to be the last surviving oysters from the grow-out site after its closure. In 1996/1997, a naturalized population was established in Lake Lockhart, New Brunswick with oysters derived from the last two hatcheries left in Nova Scotia (Port Medway and Lunenburg). The Lake Lockhart population has grown rapidly and undergone two generations in the wild. It is the largest naturalized European oyster population in the Maritimes region of Canada. The Pacific Coast stock, sampled for comparison purposes, is a hatchery maintained stock of a mixed and poorly documented origin. Transfers have occurred in the last 20 years

with individuals originating from California, Scotland and Maine probably via Nova Scotia.

#### *Amplification and Visualization of Microsatellites*

To prepare the samples for DNA extraction, frozen tissue was thawed at room temperature. Both frozen and ethanol-preserved samples were rinsed in distilled water to remove residual salt from the tissue. DNA was extracted using DNeasy Tissue Kits, following the manufacturer's instructions (Qiagen cat #69506).

Polymerase Chain Reaction (PCR) was used to amplify 5 variable tandem repeat loci (microsatellites): 4 dinucleotides *OeduU2*, *OeduT5*, *OeduO9*, and *OeduJ12*, and 1 tetranucleotide, *OeduH15* developed at IFREMER (Launey 1998, Launey et al. 2002). Primers were labeled at the 5' end with a fluorescent dye. Eight microliters of reaction mix (10  $\mu$ M of each primer, 15 mM  $MgCl_2$ , 1  $\mu$ l 10  $\times$  buffer, 1  $\mu$ l dNTP mix, and 0.5 u *Taq* polymerase) was added to 20–40 ng of DNA from the extraction. PCR amplification was performed using a MJ Research Dyad thermocycler (model PTC-220). A touchdown PCR was used to increase the specificity of the reaction (cycling conditions: an initial denaturation step at 94°C for 2 min, followed by 1 cycle at target annealing temperature ( $T_a$ ) +5°C for 1 min, with subsequent decrease of 1°C/cycle for the next 4 cycles, with the remaining 25 cycles at  $T_a$  and a final 5-min extension at 72°C).

PCR products were electrophoresed on a 0.075 mm 5% denaturing polyacrylamide gel with an internal size standard (Megabase ET-400, Amersham-Pharmacia cat #25-0205-01) added to each lane. Electrophoresis and visualization of alleles were performed using a MJ Research BaseStation Fragment Analyzer. Alleles were assigned scores (size in base pairs) by comparing them to the internal size standard using Cartographer software (Cartographer v 1.2.6, MJ Research). To standardize scores obtained in this study with Launey et al (2002)'s study, 12 individuals genotyped at the IFREMER station in France on a Licor platform were selected for their wide range of allele sizes at each locus. These individuals, were also genotyped on the MJ Research BaseStation in Canada. The scores obtained from the two different platforms were compared and adjustments were made if required. Only two

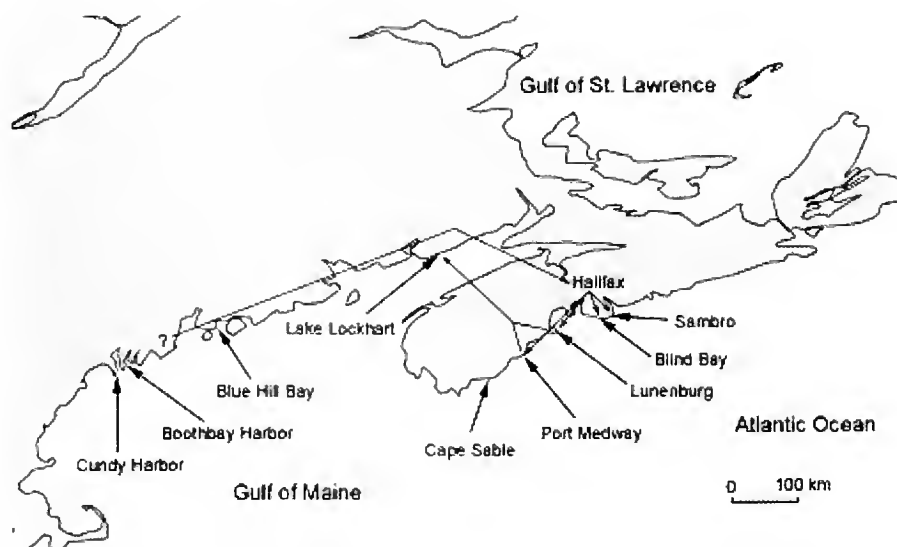


Figure 1. Map showing sampling locations and transfers of *Ostrca edulis* (long thin arrows indicate transfers to and within Nova Scotia and to New Brunswick)

microsatellite markers (*OeduJ12* and *OeduH15*) could be unambiguously standardized over the complete range of allele sizes.

#### Data Analysis

Observed and unbiased expected heterozygosities, F-statistics and genetic distances were calculated with the program GENETIX (Belkhir et al. 1996–2004) and the program CONTRIB (Petit et al. 1998) was used to calculate allelic richness corrected for differing sample size. The software MICRO-CHECKER (Van Oosterhout et al. 2004) was used to check for the potential presence of null allele and genotyping errors in the data set. PHYLIP (Felsenstein 1993) was used for drawing phylogenetic trees among the various populations. Phylogenetic analyses were based on all five loci when looking at genetic differentiation among the North American collections alone, but were based on two loci (*OeduH15* and *OeduJ12*) when using the European populations genotype data collected in an earlier work (Launey 1998, Launey et al. 2002).

In addition, 10 oysters from each of the three Maine collections were selected at random and removed from the database. These 30 oysters were then treated as an “unknown” sample to see to which populations they would be assigned on the basis of their genetic profiles using GENECLASS (Cornuet et al. 1999). The computation is based on a likelihood method using a Bayesian approach and gives for each individual a list of populations for which the “probability of belonging” is larger than a chosen threshold. We used the default probability of 0.01.

Lastly, DNA marker based pedigree reconstruction among the various collections of oysters was undertaken using Pedigree 2.2, a much expanded version of the pairwise score method (Smith et al. 2001, Butler et al. 2004), accessible online at <http://herbinger.biology.dal.ca:5080/Pedigree>. This approach to first generation pedigree reconstruction uses a highly efficient Markov Chain Monte Carlo algorithm to sample the space of possible partitions and to maximize an overall partition score based on the logarithm of pairwise likelihood ratios of being full-sib or unrelated. A partition is an allocation of all individuals into putative groups. Here, the individuals in the various oyster collections were allocated into putative kin groups, where a kin group contains individuals that appear related based on the DNA marker data but without imposing a particular type of relationship among the individuals. Individuals within kin groups are typically a mixture of full-sibs and half-sibs. This analysis was performed only on individuals with at least four loci genotyped and used the following MCMC parameters: 3000,000 iterations, annealing temperature of

10 and a weight of 1, because the various data sets did not appear to contain large family groups (see help manual available at <http://herbinger.biology.dal.ca:5080/Pedigree>).

Most pedigree reconstruction algorithms tend to assemble, by chance, unrelated individuals into small artefactual groupings (Smith et al. 2001, Butler et al. 2004). To test whether the kin group partitions generated for each oyster collections may contain at least a few truly related individuals, 100 genotype randomization trials were performed, followed by kin group reconstruction for each data set. This created 100 sets of unrelated individuals sampled from populations with the same genotypic frequencies as in our original datasets. The overall significance (*P* value) of a kin partition was evaluated by the proportion of the 100 randomized trials with a partition score as high or higher than the observed score.

## RESULTS AND DISCUSSION

#### Allelic Variability, Allelic Richness Corrected for Sample Size and Allelic Frequencies

The five microsatellite loci used in this study were all found to be highly polymorphic. Allelic richness is highly dependent on effective population size (Nei et al. 1975) and should be a good indicator of past demographic changes (Petit et al. 1998). The number of alleles found at each locus ranged from 14 (*OeduH15*) to 29 (*OeduU2*), with a mean of 22.8.

Table 1 compares the observed number of alleles in the Canadian, Maine and Northern European populations overall. The observed number of alleles was lower in the Maine populations compared with the Northern European populations (data from Launey, 1998) at four of the five loci used in this study. The loss ranged from three alleles (*OeduH15*) to five alleles (*OeduU2*). The Canadian populations showed an even greater loss of allelic diversity, ranging from three alleles (*OeduH15*) to nine alleles (*OeduO9*), when compared with the Northern European populations. This was true despite the fact that the sample size for the Canadian populations was much larger than the Maine and European populations.

When the Canadian populations were compared with the Maine population, there was a loss of alleles at 3 loci: 2 alleles at *OeduU2*, 6 at *OeduT5*, and 5 at *OeduO9*. There was no loss of alleles at *OeduH15*, and a “gain” of an allele at *OeduJ12*. This “gain” is likely an artifact caused by the lower number of individuals from Maine.

Direct comparisons of allelic richness between populations was

TABLE 1.

Number of individuals genotyped and the number of observed alleles for the combined Canadian and Maine populations. Data for the Northern European populations of *Ostrea edulis* were obtained from Launey (1998).

Locus	Canadian Populations		Maine Populations		North European Populations (Launey, 1998)	
	Number of Individuals	Observed nb. of Alleles	Number of Individuals	Observed nb. of Alleles	Number of Individuals	Observed nb. of Alleles
<i>OeduU2</i>	356	26	139	28	254	33
<i>OeduT5</i>	427	21	239	27	254	26
<i>OeduH15</i>	403	14	236	14	254	17
<i>OeduO9</i>	426	12	246	17	254	21
<i>OeduJ12</i>	425	23	251	22	254	27
Average		19.2		21.6		24.8

difficult because of the large range in sample sizes (30–148), because larger samples will have a greater chance of including rare alleles. The program CONTRIB was used to correct for differences in sample size. Table 2 illustrates the amount of genetic diversity present in each of the sampled populations by showing the actual and corrected numbers of alleles at each locus. The NS hatchery stocks had a mean corrected number of alleles ranging from 9.91 (Port Medway) to 10.32 (Lunenburg). The British Columbia hatchery stock was slightly more diverse (11.20), and Maritimes naturalized populations varied from 8.88 (small "naturalized" Blind Bay group, which shows the lowest mean number of corrected number of alleles) to 12.81 (Lake Lockhart) alleles. The Maine populations ranged from 12.43 (Blue Hill Bay) to 15.29 (Boothbay Harbor).

At each locus, evidence of some genetic erosion can be seen with smaller number of alleles observed in the Canadian collections. Compared with the 2 larger Maritimes naturalized populations (Sambro, NS and Lake Lockhart, NB) and to the Maine naturalized populations (Cundy's Harbor, Boothbay Harbor and Blue Hill Bay), the combined Nova Scotian hatchery stocks (Lunenburg, Port Medway and Cape Sable) showed the lowest number of alleles at all loci, with a mean of 13.6 alleles for all loci. The Maritimes naturalized populations showed a mean of 17.6 alleles and the Maine populations a mean of 21.6 alleles. Nevertheless, the actual number of alleles at each locus was still relatively high in the Nova Scotia hatchery stocks.

Some degree of on-going genetic erosion is therefore taking place in the artificially propagated populations of *Ostrea edulis*, but it would appear that as of 2002–03 there is still a substantial level of genetic diversity in the Canadian collections, despite the fact that these populations have been isolated from both their ancestral European and Maine populations and have been propagated in hatcheries for several generations. As a comparison, in *O. edulis*

French populations selected for *Bonamia* resistance, the mean number of alleles was reduced from 19.8 (natural population) to a range of 5.4–12.8 (selected populations) in two generations (Launey et al. 2001). The Canadian populations seem to have experienced considerably less erosion of allelic richness in 6–7 generations since their importation from Maine. In other words, genetic erosion could have been more drastic than was revealed in this study.

Genetic erosion is a common concern for hatchery stocks. An estimation of the breeding number of oysters in hatchery *Crassostrea virginica* MSX resistant lines varied from 4–16 (Hedgecock et al. 1992). Hatchery-propagated Pacific oysters have also lost alleles in three generations and one hatchery stock showed a per-generation effective population size of 9 oysters (Hedgecock & Sly 1990). Boudry et al. (2002) showed that effective population size was strongly reduced compared with census size in *Crassostrea gigas* because of unbalanced parental contribution. For *O. edulis*, Launey et al. (2001) estimated that the effective size of oyster populations selected for *Bonamia* resistance was very low, between 3 and 20 at the most. In the present study, effective population size  $N_e$  could not be determined but was probably higher than in the populations studied by Launey et al. (2001). Alvarez et al. (1989) and Saavedra and Geera (1996) previously reported the number of effective *O. edulis* broodstock to be 6–10 and 3–4 from mass spawning tanks containing 60 and 120 animals respectively. This evidence shows that *O. edulis* is a species susceptible to founder and bottleneck effects in the hatchery environment and that genetic erosion has to be carefully monitored.

In this study, the allelic richness corrected for unequal sample size clearly indicated that the Lake Lockhart population was the most diverse in the Canadian collections. This is probably because of the fact that it is a relatively large, truly naturalized population. Although sampled from the wild, the Blind Bay population did not

TABLE 2.

Sample size ( $n$ ) and total number of alleles (= corrected number for sample size) per locus for each population of *Ostrea edulis*. NS hatcheries, Maritimes naturalized and Maine naturalized combined for comparison. Hatcheries: BC\_PAC: Pacific Coast, BC, NS\_LUN: Lunenburg, NS, NS\_MED: Port Medway, NS, NS\_CAS: Cape Sable, NS, Maritimes naturalized: MAR\_BLB: Blind Bay, NS, MAR\_SAM: Sambro, NS, MAR\_LLO: Lake Lockhart, NB, Maine naturalized: MNE\_CUH: Cundy Harbor, ME, MNE\_BOH: Boothbay Harbor, ME, MNE\_BHB: Blue Hill Bay, ME.

Population	n <sup>(1)</sup>	Number of Alleles					Average
		OeduU2	OeduT5	OeduH15	OeduO9	OeduJ12	
BC_PAC	30	16–16	12–11.93	8–8.00	9–9.00	11–11.00	11.2–11.20
NS_LUN	68	14–12.62	12–11.39	9–7.86	9–8.16	13–11.59	11.4–10.32
NS_MED	39	15–13.95	13–11.44	8–7.89	9–8.26	9–8.02	10.8–9.91
NS_CSA	125	18–14.42	17–11.85	7–6.87	10–8.37	16–8.92	13.6–10.09
Total NS hatcheries	233	18	17	9	11	20	13.6
MAR_BLB	36	11–10.91	12–12.00	8–7.77	7–6.86	7–6.84	9.0–8.88
MAR_SAM	57	15–14.21	17–14.72	10–9.37	12–10.53	15–11.53	13.8–12.07
MAR_LLO	148	24–18.44	18–14.63	11–9.24	11–9.05	18–12.70	16.4–12.15
Total Maritimes naturalized	188	25	19	12	12	20	17.6
MNE_CUH	100	22–20.26	23–16.39	14–11.88	16–12.10	19–15.02	18.8–15.13
MNE_BOH	89	22–19.84	25–18.58	11–10.14	12–10.45	21–17.44	18.2–15.29
MNE_BHB	65	21–17.42	16–13.89	9–8.71	12–9.98	15–12.16	14.6–12.43
Total Maine naturalized	257	28	27	14	17	22	21.6

(1) The sample size is given here as an indication only; only all individuals amplified at each locus. The BC\_PAC collection is the smallest collection; hence corrected number of alleles is equal to the actual number of alleles for all loci but T5 where for the MAR\_BLB collection, 29 samples only amplified at that particular locus.



TABLE 3.

Statistics per population of *Ostrea edulis*: sample size (n), non biased expected heterozygosity  $H_e$  (Nei, 1978), observed heterozygosity  $H_o$ , and Hardy-Weinberg Equilibrium deviations estimated by  $F_{is}$ .

Population	n	$H_e$	$H_o$	$F_{is}$					
				All loci	<i>OeduU2</i>	<i>OeduT5</i>	<i>OeduH15</i>	<i>OeduO9</i>	<i>OeduJ12</i>
BC_PAC	30	0.860	0.827	0.040	0.134	-0.012	0.044	-0.057	0.079
NS_LUN	68	0.810	0.670	0.175	0.003	0.007	0.459	0.130	0.335
NS_MED	39	0.786	0.736	0.064	0.002	-0.094	0.199	-0.166	0.454
NS_CSA	125	0.795	0.695	0.121	0.059*	0.024	0.382	-0.083	0.290
MAR_BLB	36	0.773	0.678	0.124	-0.063*	-0.018	0.406	0.052	0.357
MAR_SAM	57	0.855	0.706	0.176	-0.018	0.019	0.485	0.087	0.344
MAR_LLO	148	0.821	0.707	0.138	0.076	-0.024	0.547	-0.060	0.190
MNE_CUH	100	0.876	0.720	0.179	0.051	0.056	0.585	0.049	0.159
MNE_BOH	89	0.889	0.793	0.110	-0.031	0.017	0.447	-0.019	0.155
MNE_BHB	65	0.854	0.753	0.119	0.081	-0.007	0.420	-0.079	0.202

\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$

appear as variable as the two other naturalized Nova Scotian populations. This is a probable indication that it is not a truly naturalized population. Divers at this site did not find any juvenile oyster or any sign of recruitment. The few large oysters found there were probably the last survivors of the previous cultivated oysters. The five remaining Canadian populations appeared similar, with possibly a slightly higher diversity in the Pacific Coast stock. Repeated transfers have occurred in the last 20 years with individuals originating from different populations in California, Scotland and Maine, possibly explaining the higher diversity in this hatchery stock.

The allelic profiles for each locus showed multimodal distributions both in the Maine population and the Canadian population as they did in Europe (Launey 1998). Only the distribution for *OeduO9* was close to a normal distribution in a European sample of 507 individuals (Launey 1998). There was no major discontinuity in the distributions and increments corresponded to the repeat length for a given locus (dinucleotide for all loci except *OeduH15*, which is a tetranucleotide locus). However the various collections showed differences in the abundance of rare alleles (frequency  $<10\%$ ). A high level of rare alleles (87% average over 5 loci) characterized the Maine population, whereas the Maritimes naturalized populations and hatchery stocks showed lower levels of 73% and 69% respectively. The hatchery populations lost 5, 2, 7, 8 and 2 rare alleles at loci *OeduU2*, *OeduH15*, *OeduT5*, *OeduO9* and *OeduJ12* respectively compared with the naturalized popula-

tions. These results are usually observed when a population experiences a bottleneck event but may also reveal subsequent genetic erosion.

#### Heterozygosity

The observed heterozygosity ( $H_o$ ) was lower than the expected heterozygosity ( $H_e$ ) for all populations (Table 3), a typical observation in bivalves (Hedgecock et al. 1992, Hedgecock & Okazaki 1984). The difference between  $H_e$  and  $H_o$  was smallest in the British Columbia and Port Medway samples. This may be a result of a breeding program for certain traits that has led to the incidental selection of heterozygotes, or may possibly be an artifact of the sampling procedure, because the sample sizes for these populations were both relatively small.

When looking at the hatchery stocks (Table 3), levels of heterozygosity were not as dramatically reduced as allelic richness was. The average observed heterozygosity ( $H_o$ ) for each population ranged from 0.670 (Lunenburg) to 0.827 (British Columbia) and no apparent association between the observed heterozygosity and the origin of the samples (hatchery vs. naturalized) was observed. Similar results have been reported for many cultivated populations (Herbinger et al. 2003); in particular an earlier study on *Crassostrea gigas* showed that the number of alleles was significantly reduced but heterozygosity was retained in hatchery stocks compared with naturalized populations (Hedgecock & Sly 1990, Herbinger et al. 2003).

TABLE 4.

$F_{ST}$  values for population pairs of *Ostrea edulis* according to Weir and Cockerham (1984).

Population ( $F_{ST}$ )	BC_PAC	NS_LUN	NS_MED	NS_CSA	MAR_BLB	MAR_SAM	MAR_LLO	MNE_CUH	MNE_BOH
NS_LUN	0.038								
NS_MED	0.049	0.027							
NS_CSA	0.048	0.019	0.021						
MAR_BLB	0.057	0.018	0.026	0.007 <sup>NS</sup>					
MAR_SAM	0.041	0.028	0.050	0.024	0.029				
MAR_LLO	0.033	0.011	0.022	0.011	0.013 <sup>NS</sup>	0.021			
MNE_CUH	0.024	0.046	0.054	0.046	0.054	0.038	0.036		
MNE_BOH	0.019	0.043	0.051	0.045	0.056	0.035	0.035	0.003 <sup>NS</sup>	
MNE_BHB	0.038	0.037	0.040	0.039	0.048	0.028	0.026	0.023	0.019

<sup>NS</sup>:  $P > 0.001$

The  $F_{IS}$  values for the five loci, all populations combined, ranged from  $-0.094$  (*OeduT5*) to  $+0.585$  (*OeduH15*) (Table 3), which represents a large range of values. The estimated  $F_{IS}$  values varied much less among samples (from  $+0.040$  to  $+0.179$ ) than among loci. This result indicates that inbreeding is not the sole explanation for the heterozygote deficiencies. All populations showed mostly positive  $F_{IS}$  values revealing deficits in heterozygosity, but this was really mostly prevalent for loci *OeduH15* and *OeduJ12* (Table 3). Such observations are common in bivalves (Zouros & Foltz 1984) and the same pattern was observed in the European populations (Launey 1998). This is likely due mostly to the presence of null alleles (alleles that are not amplifying, possibly caused by a mutation in the primer site). Indeed, MICRO-CHECKER revealed the probable presence of null alleles for *OeduH15* and *OeduJ12* in nearly all populations. Launey (1998) also hypothesized that null alleles were present for the locus *OeduH15*. *O. edulis* natural populations have high fecundity and potentially large dispersal of gametes and larvae, and so should follow the HWE assumptions for panmixia. In the context of the hatchery-propagated populations, heterozygotes deficit could possibly also reflect blind selection effects, along with small reproductive population size and inbreeding (Hedgcock 1994). However, given the relative similarities of the  $F_{IS}$  values observed in naturalized and hatchery population, it would seem that the presence of null alleles at loci *OeduH15* and *OeduJ12* are the main reasons for the observed heterozygote deficiencies here.

#### Genetic Differentiation Between European Oyster Populations

The genetic differentiation between populations revealed by the pairwise  $F_{ST}$  values are displayed in Table 4. Most of the  $F_{ST}$  values under 0.027 were not significant and indicated negligible genetic differentiation. This can be illustrated graphically with an unrooted tree constructed using the coancestry distances (Reynolds et al. 1983) (Fig. 2a). The Maine populations clustered together with the Pacific Coast population, probably reflecting the founding events that led to the establishment of the Pacific Coast population through the transfer of oysters from California, Scotland and Maine. Despite their recent origin, the Maritimes populations (Port Medway, Lunenburg, Lake Lockhart and Cape Sable) clustered together and were quite divergent from the Maine populations. The intermediate position of the Sambro oysters possibly reflects the fact that they are an older renaturalized population consisting of Maine oysters that were maintained at Dalhousie University in Halifax for several years before being released. The close clustering of the Nova Scotian populations is explained by their recent common ancestry and the exchanges of individuals that have taken place between these populations. For example, the fact that the Lake Lockhart and Lunenburg populations cluster closely together reflects the fact that the Lake Lockhart population was established using mainly oysters from Lunenburg stocks.

The Northern American flat oyster populations were compared with 5 European populations of flat oysters representing 4 subregions of the sampling realized in Launey et al. (2002), (ANa from Oslofjorden, Norway (North Atlantic); ANb from Grevelingen, Netherlands (North Atlantic); ASb from La Rochelle, France (South Atlantic); MWb from Thau Lagon, France (West Mediterranean Sea); MEb from Dubrovnik, Croatia (East Mediterranean Sea)). The analyses were based on the 2 microsatellite markers (*OeduJ12* and *OeduH15*) that could be unambiguously standardized between the genotyping systems from the Canadian and

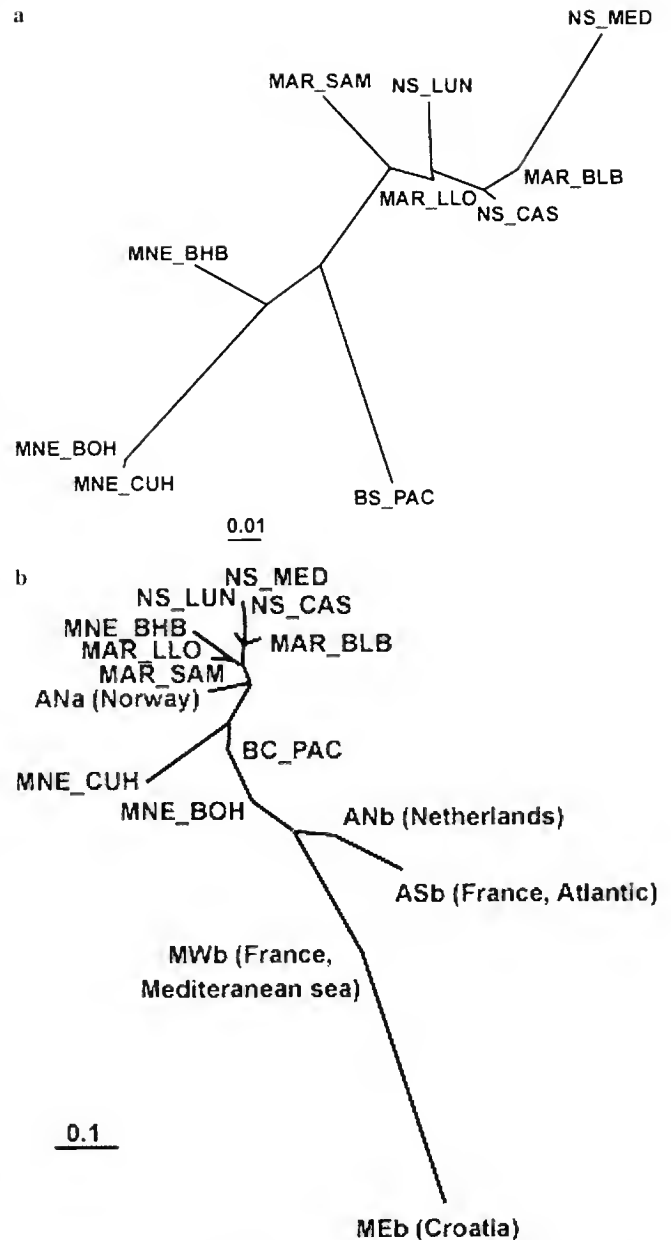


Figure 2. Unrooted Neighbor-Joining tree (Saitou & Nei 1987) obtained from coancestry genetic distances (PAC: Pacific Coast; LUN: Lunenburg; MED: Port Medway; CAS: Cape Sable; BLB: Blind Bay; SAM: Sambro; LLO: Lake Lockhart; CUH: Cundy Harbor; BOH: Boothbay Harbor; BHB: Blue Hill Bay). a. Canadian and Maine populations (5 loci) b. Canadian and North Atlantic European populations (2 loci)

French molecular laboratories. The resulting neighbor-joining tree is visualized in Figure 2b. The sample from the Netherlands (ANb) showed  $F_{ST}$  values not significantly different from 0 with 2 Maine populations (Cundy Harbour and Boothbay Harbour) confirming the Netherlands as the probable origin of the introduction of the European flat oyster on this Northern American region of Maine (Loosanoff, 1955). Although significant, the  $F_{ST}$  values between the North Atlantic sample from Norway (ANa) and each of the Northern American populations were also small. The neighbor-joining tree clearly shows that the sample from Norway (ANa) is clustering (all the bootstrap values were superior to 50%) with the

TABLE 5.

Assignment of 3 samples of 10 random *Ostrea edulis* individuals from Maine using GENECLASS (MNE\_CUH: Cundy Harbor, ME, MNE\_BOH: Boothbay Harbor, ME, MNE\_BHB: Blue Hill Bay, ME, MAR\_SAM: Sambro, NS, MAR\_LLO: Lake Lockhart, NB).

Sample Size	Number of Loci	(#) Classified with the Highest Probability in	Notes	Real Origin
10	5	(4) MNE_CUH (3) MNE_BOH also classified in MNE_CUH (1) BOH also classified in MNE_CUH and MNE_BHB (2) unclassified ( $P < 0.01$ )	No individual classified outside of Maine	MNE_CUH
10	5	(2) MNE_BOH (2) MNE_BOH also classified in MNE_CUH (2) MNE_CUH (4) unclassified ( $P < 0.01$ )	No individual classified outside of Maine	MNE_BOH
10	5	(3) MNE_CUH (1) MNE_BOH (1) MNE_CUH also classified in MNE_BHB (2) MNE_CUH also classified in MNE_BHB, MNE_BOH, MAR_LLO and/or MAR_SAM (3) unclassified ( $P < 0.01$ )	20% classified outside of Maine (2 re-naturalized populations)	MNE_BHB

samples from Nova Scotia, indicating a possible second distinct introduction from Europe. It should be noted however that this analysis is based on only two loci with probable null alleles. Hence, this should be seen as a preliminary indication only and such analysis should be extended with more loci.

#### Assignment of Maine Individuals

Assignment of the 30 Maine individuals on the basis of their genotype was overall satisfactory (Table 5) even though this was based on 5 loci, and the genetic isolation between Maine and Nova Scotia populations is fairly recent. Nine individuals could not be definitively assigned to a population but 19 were correctly identified as originating from a Maine population only and 2 were assigned to another Maine population or to a Nova Scotia naturalized population. Among those 21 individuals, 15 were assigned to the specific Maine population they originated with either the highest likelihood (8 cases) or the second highest (7 cases). It should be noted that most of the individuals from Blue Hill Bay were assigned to another population of Maine or to a renaturalized population in Nova Scotia. That particular population could have been founded with individuals originated from other parts of Maine and from California (Sam Chapman, pers. comm). In addition, Blue Hill Bay is the Maine population the least different from the Canadian stocks (Fig. 2a). The assignment results presented here show both promise as a forensics tool and some limitations. With a larger database and more microsatellite loci, more definite results could probably be achieved. Nonetheless, the present tool would seem to be able to detect illegal importation of Maine oysters that could bring the *Bonamia* disease into Nova Scotia.

#### First Generation Pedigree Reconstruction

Most of the kin groups detected in the 10 oyster collections were very small, ranging in size from 1–11 with an average of 2.5 individuals, indicating that overall, most individuals were unrelated to one another in every collection. However, at least some of these small proposed kin groups appeared to be real, because in 6 out of the 10 populations the kin group partition score, based on the real genotype data, was higher than seen in any of the 100

genotype randomization trials (Table 6). Not surprisingly, every hatchery population seemed to comprise at least a few truly related individuals (i.e., full or half-sibs). This was particularly the case for the Port Medway and Cape Sable collections. This is in agreement with the observations of Li & Hedgecock (1998) and Boudry et al. (2002) who have noted that hatchery mass spawning typically contains large, over-represented family groups. In contrast, the naturalized populations of Cundy Harbor (Maine), Booth Bay (Maine) and Sambro (Nova Scotia) did not appear to contain related individuals. The last naturalized Maine collection, Blue Hill Bay, may have contained some related individuals, but the signal from this analysis was fairly weak and the probability value was close to the 5% threshold. Some of the "naturalized" individuals in the Blind Bay (Nova Scotia) collection also appeared to be related

TABLE 6.

Kin group partition of the *Ostrea edulis* collections.

Population	n <sup>(1)</sup>	Probability of Seeing a Score as High or Higher in Genotype Randomization Trials
Hatcheries		
BC_PAC: Pacific Coast, BC	30	<1%
NS_LUN: Lunenburg, NS	68	<1%
NS_MED: Port Medway, NS	39	<1%
NS_CAS: Cape Sable, NS	123	<1%
Maritimes naturalized		
MAR_BLB: Blind Bay, NS	31	<1%
MAR_SAM: Sambro, NS	34	NS (50%)
MAR_LLO: Lake Lockhart, NB	139	<3% <sup>(2)</sup>
Maine naturalized		
MNE_CUH: Cundy Harbor, ME	78	NS (65%)
MNE_BOH: Boothbay Harbor, ME	78	NS (16%)
MNE_BHB: Blue Hill Bay, ME	64	5%

(1) Sample size

(2) Probability estimated on 1,000 randomization trials, as opposed to 100 in the other collections

to one another. This would be in agreement with the proposed explanation that this population is not truly self sustaining, and that the sampled individuals probably represented the last survivors of "escaped" cultured oysters from a grow-out site that operated there until a few years ago. The observation of truly related individuals in the Lake Lockhart (New Brunswick) collection is more surprising because this is a fairly large, self-sustaining population. To confirm this result, the genotype randomization trials were extended to 1,000 but with similar results. Partition scores as high or higher than the one observed on the real data, were only seen in 2 out of the 1,000 trials. It is unlikely that oyster sampling in this location was somehow biased with respect to family composition as it took place with bottom dredging. This observation may be an indication that a few individuals may sometimes contribute a substantial (i.e., detectable) portion of the recruitment in the large but closed Lake Lockhart population.

### CONCLUSION

The results of this study show that there is still a relatively high level of genetic diversity in the Canadian populations, but evidence of genetic erosion can be seen in the hatchery-propagated stocks. Some level of inbreeding increase occurs in hatchery populations of *Ostrea edulis* around the world, and there is evidence to suggest that excessive inbreeding can result in loss of fitness. For instance, Mallet & Haley (1983) and Naciri-Graven et al. (2000) have observed that growth performance of offspring is negatively correlated with the relatedness of their parents. Using microsatellite loci to monitor inbreeding levels would appear to be warranted.

Increasing the number of loci available for use in *O. edulis* would be useful for continuing and future studies of genetic diversity and population structure in this species and for forensics studies given the presence of *Bonamia* in Maine and its absence so far in Nova Scotia and New Brunswick. Increasing sample size will also improve our ability to detect population and family structure more effectively.

Hatchery production for aquaculture allows the development of genetically improved strains but, simultaneously, loss of genetic diversity and potential inbreeding increase and inbreeding depression can have adverse effects. To manage inbreeding, it is impor-

tant to use pedigree information when producing the next generation. Because of the reduced number of effective broodstock in mass spawning, where only a limited number of individuals contribute most of the offspring to the next generation, it may be wise to increase genetic variability in hatchery populations by maximizing the number of *O. edulis* broodstock used to produce larvae (at least 50 per lot, preferably 100) and pooling offspring from multiple spawning groups (i.e., multiple lots). The genetic impact of grading larvae and discarding the small ones, a common hatchery practice, should be evaluated. This practice may compound genetic erosion without really achieving any growth gain. The possibility of introducing individuals from naturalized populations at regular intervals to maintain genetic diversity and prevent further erosion of genetic diversity in Nova Scotia hatchery stocks should be evaluated as well. Outbreeding of *O. edulis* should be carried out within Maritimes populations as opposed to more distant populations to reduce the possibility of disease transfer and also to avoid the introgression of nonadapted genes into the actual stocks. In particular, history shows that only after a 30-year adaptation to Maine's cold environment did introduced European oysters survive in Nova Scotia's marginal winter conditions.

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## TISSUE DISTRIBUTION AND HEMOLYMPH ACTIVITY OF SIX ENZYMES IN THE AMERICAN LOBSTER (*HOMARUS AMERICANUS*): POTENTIAL MARKERS OF TISSUE INJURY

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**ABSTRACT** Biochemistry panels are used to help identify tissue injury (e.g., because of inflammation, trauma or hypoxia) in human and veterinary medicine in part, by detecting increased enzyme activity in serum or plasma after release from damaged tissues. To determine if a similar approach can be used in *Homarus americanus*, activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), glutamate dehydrogenase (GDH), sorbitol dehydrogenase (SDH), amylase (AMY) and lipase (LIP) were measured in tissue homogenates of heart, hepatopancreas, abdominal muscle, proximal intestine, antennal gland, hemocyte lysate supernatant and hemolymph plasma and serum. Activities of ALT and AST were significantly higher in serum than plasma, which was attributed to release of enzymes from hemocytes during coagulation. Reference intervals calculated for plasma enzyme activity at ambient holding temperatures of 2 °C to 4 °C and 15 °C were quite similar. Plasma enzyme activity was not a sensitive test for detecting infection with *Aerococcus viridans* (gaffkemia) during an experimental trial.

**KEY WORDS:** lobster, *Homarus americanus*, enzyme, hemolymph

### INTRODUCTION

The lobster fishery is a multimillion-dollar industry in Atlantic Canada and the northeastern USA. The landed value of the ~42 K mt harvest in Atlantic Canada in 2004 is estimated at ~\$525,077 K (Cdn)<sup>1</sup>. The overall economic impact to the region of this vital industry is far greater. Although much of the harvest is sold or processed immediately, a portion is held live, for several weeks to months, in lobster pounds for sale as live product at a later date. Significant losses to disease can occur during these periods. Consequently, being able to assess the health status of these animals, as a means of selecting those best suited for storage, is very beneficial.

Current methods of health assessment in *Homarus americanus* (H. Milne Edwards 1837) and many other crustaceans include: physical examination to determine vigor; total hemocyte counts (THCs); estimating total hemolymph protein concentration using refractometry (TP<sub>n</sub>) and hemolymph culture to detect infective agents (bacteria, protozoa). Evaluation of the cell-free fractions of hemolymph, i.e., plasma and serum, in *H. americanus* has included electrolyte and metabolite levels (Mercaldo-Allen 1991, Mercaldo-Allen et al. 1994) and enzyme activity (Speare et al. 1996). Evaluation of hemolymph enzyme activity in mussels and oysters has been suggested as a nonlethal way of assessing overall health, and possibly immune function, as in human and veterinary medicine (Culloty et al. 2002, Gustafson et al. 2005).

Clinical enzymology can be thought of as the “application of the science of enzymes to the diagnosis of disease” (Moss & Henderson 1999). The level of enzyme activity detected in the blood is related to the rate of release or production of enzyme(s) from tissues and their clearance from the circulation (Moss & Henderson 1999). Some enzymes are relatively tissue specific, being found in only one or a few tissues, whereas others can be more widely distributed among several tissues (Duncan et al. 1994a, Kramer & Hoffmann 1997, Moss & Henderson 1999). There will usually be low levels of enzymes in the blood caused by steady-state release from tissues. These values are used to calculate

reference intervals, or “normal ranges,” for a defined (age, sex, race, geographic distribution, disease status, etc.) population. These reference intervals are then used as a basis for comparison with individuals or groups under study. When the tissue is injured (trauma, toxins, inflammation, hypoxia) the cellular integrity will be compromised and more enzyme will be released into the circulation, exceeding the upper limit of the previously established reference interval (Kramer & Hoffmann 1997, Moss & Henderson 1999). The degree of increase will be determined by the amount of tissue injured, the severity of the injury, and the clearance rate of the enzyme from the circulation.

The purpose of this investigation was to determine if the principles of clinical enzymology could be applied to *H. americanus*. This required: (1) determining the tissue distribution of a selected group of enzymes; (2) determining the preferred sample type, plasma or serum; (3) establishing hemolymph reference intervals for enzyme activities in a defined population and (4) examining if hemolymph enzyme activity was affected by a systemic bacterial infection, gaffkemia.

The activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT), sorbitol dehydrogenase (SDH), glutamate dehydrogenase (GDH), amylase (AMY) and lipase (LIP) was determined in homogenates of hepatopancreas, heart, abdominal muscle, proximal intestine, antennal gland, hemocyte lysate supernatant (HLS) and hemolymph plasma and serum. Enzymes were selected for the biochemistry panel based on the availability of commercial diagnostic test kits compatible with an automated biochemistry analyzer and their known or presumed presence in hepatopancreas and muscle (Claybrook 1983, Biesiot & Capuzzo 1990)—tissues similar to those contributing significantly to serum and plasma enzyme activity in vertebrates (Kramer & Hoffmann 1997, Moss & Henderson 1999). Clotting in *H. americanus* and other crustaceans involves hemocyte lysis (Martin & Hose 1995). Consequently, HLS was evaluated for its possible contribution to serum enzyme activity. The proximal intestine, antennal gland, and heart were included for completeness. Gaffkemia, a generally fatal infectious disease caused by the Gram positive bacterium *Aerococcus viridans*, can cause significant economic losses in the industry (Hastein & Roald 1977, Stewart 1980, Menard & Myrand 1987). Early detection could provide critical informa-

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tion for management decisions and help minimize postharvest losses.

## MATERIALS AND METHODS

### *Animals and Holding*

#### **Tissue Enzyme Distribution Study**

Four (3 female, 1 male) hard shell, southwest Nova Scotia inshore lobsters weighing 404–499 g were obtained from a commercial pound (Clearwater Fine Foods Inc., Halifax, NS, Canada). The animals were held in a recirculating artificial sea water (ASW) (Instant Ocean, Aquarium Systems Inc., Mentor, OH, USA) system maintained between 2°C to 4°C for up to 12 d. Lighting was set at a 14 h/10 h low light (<3 lux)/dark cycle. Animals were not fed during this period.

#### **Establishment of Reference Intervals and Gaffkemia Trial**

Thirty male southwest Nova Scotia offshore lobsters weighing 597–791 g were obtained from a commercial source (Clearwater Fine Foods Inc., Halifax, NS, Canada). The animals were held in a recirculating ASW (Instant Ocean, Aquarium Systems Inc., Mentor, OH, USA) system maintained between 2°C to 4°C for 9 d then moved to a second recirculating ASW system at 15°C. Lighting was set at a 14 h/10 h low light (<3 lux)/dark cycle. Animals were offered a pelleted lobster diet (Castell Aquaculture Nutrition Consulting, St Andrews, NB, Canada) every other day when at 15°C.

### *Biochemical Analyses*

All samples for biochemistry panels were analyzed on the Hitachi 917 automated biochemistry analyzer (Roche Diagnostics Corporation, Indianapolis, IN, USA) within three hours of collection. Assay kits for GD, AST, ALT and total protein (TP) were obtained from Roche Diagnostics Corporation, Indianapolis, IN, USA. Assay kits for LJP and AMY were obtained from Diagnostic Chemicals Limited, Charlottetown, PE, Canada.

Assay precision was determined by measuring enzyme activity in 20 aliquots of two samples, one with high enzyme activity and one with low activity. The coefficient of variation (CV) was calculated for each sample.

### *Tissue Enzyme Distribution Study*

Hemolymph samples were collected (22 G needle) from the ventral sinus 2 d after arrival as part of a health screen. A 0.5-mL sample was collected into 4.5 mL of formalin-containing anticoagulant (28.4 g/L NaCl, 8.7 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 5.5 g/L  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 1.5 g/L  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.7 g/L KCl, 0.5 mL Tween 80, 1.25 mL/L stock [37% to 40%] formaldehyde solution, pH 7.6, Stewart et al. 1967) for determination of THC's using a Neubauer hemocytometer. Whole hemolymph (no anticoagulant) was collected for  $\text{TP}_{\text{ti}}$  estimation using a temperature compensated refractometer (Shilac ATC PUR1410, Japan) and inoculation of culture media to detect *A. viridans* (0.5 mL in 4.5 mL phenyl ethyl alcohol [PEA] broth at 28°C for 48 h, in duplicate, Stewart 1972) and *Anophryoides haemophila* (0.5 mL into 4.0 mL of modified ciliate culture medium at 4°C for 7 d, Messick & Small 1996). Pleopods were collected for molt staging (Aiken 1973). Animals were processed after confirmation of negative culture results and  $\text{THC} > 10 \times 10^9$  cells/L and  $\text{TP}_{\text{ti}} > 50$  g/L.

Immediately prior to processing, a terminal hemolymph col-

lection (approximately 30 mL/lobster) was performed to obtain hemocyte pellets for HLS preparation. The supernatant (plasma) was removed after centrifugation ( $\times 3300$  g, 15 min at 4°C) and the pellet rinsed with sterile distilled water. Lobsters were euthanized by severing the ventral nerve cord in front of the walking legs. Samples of hepatopancreas, heart, antennal gland, proximal intestine and abdominal muscle were collected and immediately placed on ice. The contents of the intestinal lumen were removed by gentle rinsing with distilled water. Tissues ( $\leq 0.5$  g) were then minced with a scalpel blade after addition of 5 mL of sterile distilled water, transferred to a glass piston homogenizer and ground until no large particles remained. Samples were left to lyse, refrigerated, for 1 h. Tissue homogenate supernatants were prepared by centrifugation ( $\times 15,000$  g, 20 min. at 4°C) and assayed for enzyme activity and TP (biuret method) concentration. Enzyme activity per gram of total protein was calculated to standardize the results among tissues.

### *Comparison of Plasma and Serum Samples*

Hemolymph was collected (22 G needle, no anticoagulant) from the ventral sinus of the four lobsters used for the tissue enzyme distribution study. Plasma was prepared from one 1.8 mL aliquot. Serum was prepared by allowing refrigerated 1.8 mL aliquots to clot. After 5 h and 22 h, the samples were broken up and centrifuged ( $\times 5000$  g, 5 min, at 4°C) and the supernatants collected and submitted for biochemistry panels.

### *Establishment of Reference Intervals*

Hemolymph samples for THC's,  $\text{TP}_{\text{ti}}$ , biochemistry panels and inoculation of PEA were collected from the ventral sinus of 30 male, hardshell lobsters 2 d and 6 d after their arrival (2°C to 4°C) and again after being held at 15°C for 4 d. Pleopods for molt staging (Aiken 1973) and hemolymph for ciliate medium (Messick & Small 1996) inoculation were only collected at the first sampling. Reference intervals were calculated for the latter two sample collections using results from intermolt animals with  $\text{THC} > 5 \times 10^9$  cells/L and negative hemolymph cultures.

### *Gaffkemia Infection Trial*

#### ***Aerococcus viridans* Inoculate Preparation**

A thawed aliquot of a first passage subculture of a field isolate of *A. viridans* Type 3 (api 20 Strep, bioMérieux Canada Inc., St. Laurent, PQ, Canada), stored at -80°C in 2% skim milk culture broth (Bacto skim milk, DIFCO Laboratories, Detroit, MI), was grown on sheep blood (5%) agar (BA) (Oxoid, Inc. (Canada), Nepean, ON) at 28°C for 24 h and used to inoculate 25 mL of sterile trypticase soy broth (TSB). Broth cultures were incubated overnight at 28°C and the optical density was read at 420 nm. The suspension was washed ( $\times 3600$  g, 10 min, at 4°C) twice with sterile 3% NaCl and an aliquot diluted to an expected concentration of  $2 \times 10^6$  colony forming units (CFU)/mL. Colony counts were performed on  $\times 25$   $\mu\text{m}$  aliquots of serial dilutions cultured on BA at 28°C after 48 h of incubation to confirm inoculate dose.

The supernatant from a 24 h TSB culture of *A. viridans* was submitted for a biochemistry panel.

### **Experimental Protocol**

Thirty male lobsters were acclimatized at 2°C for 10 d and then transferred into a new system at 15°C for 7 d. Hemolymph samples



were collected from the ventral sinus for determination of THCs, TP<sub>tr</sub> and biochemistry panels and a few drops of whole hemolymph were used to inoculate BA, incubated at 28°C, for detection of *A. viridans* bacteremia. Twenty lobsters with THC >  $10 \times 10^9$  cells/L, and negative culture results were selected and randomly assigned into treatment and control groups. Control group lobsters were moved into a replicate system in the same room. Lobsters in the treatment group were inoculated (intra-abdominal sinus injection, 25 G needle) with *A. viridans* at  $\sim 1 \times 10^6$  CFU/kg. Control group lobsters received a similar volume of sterile 3% NaCl. One sentinel lobster (no injection) was kept in each tank.

Hemolymph was collected daily for determination of THCs, TP<sub>tr</sub>, biochemistry panels and BA inoculation. A direct smear (DS) of hemolymph was also made and examined by direct light microscopy to detect bacteremia. The study was terminated after 5 d because all treatment group lobsters were bacteremic on DS exam.

Lobsters were euthanized by KCl injection (Battison et al. 2000). Post mortem examinations were performed. The hepatopancreas and antennal gland were sampled with sterile swabs (BBM CultureSwab Plus, Becton Dickinson, Basel, Switzerland) for culture on BA at 28°C for 48 h. Representative samples of hepatopancreas, proximal intestine, antennal gland, muscle, gonad and ventral nerve cord were collected and fixed in 1G:4F fixative (Howard & Smith 1983) and processed routinely for hematoxylin and eosin staining of paraffin sections.

#### Statistical Analyses

Statistical analyses were performed using the Minitab release 13 software package (Minitab Inc., State College, PA, USA) and Microsoft Excel 2002 (Microsoft Corporation, USA).

## RESULTS

#### Precision Study

Precision results at the lower activity levels found in plasma were generally moderate to poor, ranging from 5% to 24% (Table 1). The value for SDH was very high (245%) as all but one of the 20 replicates returned a value of 0 U/L. Precision at the higher activity levels found in serum was acceptable, being below or near 5% in all cases.

TABLE 1.

Precision results for six enzymes\* in plasma or serum, representing lower and higher activity levels respectively, in *Homarus americanus*. An aliquot of each sample was assayed 20 times.

	AMY	LIP	SDH	ALT	AST	GD
Plasma						
Range (U/L)	0–0	9–15	0–8	14–33	11–14	11–13
CV**	–	16%	245%	24%	9%	5%
Serum						
Range (U/L)	0–0	6–8	0–0	220–224	111–129	13–20
CV	–	7%	–	2%	4%	7%

\* Abbreviations: AMY, amylase; LIP, lipase; SDH, sorbitol dehydrogenase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GD, glutamate dehydrogenase.

\*\* CV, coefficient of variation = standard deviation/mean \* 100.

#### Tissue Enzyme Distribution

The hepatopancreas was the exclusive source of AMY in all but one lobster where it was also detected in the proximal intestine (Table 2). Moderate to high levels of AST and ALT were found in all tissues with the heart containing the most activity (Table 2). The highest GD activity was found in the heart with only low to moderate levels recovered from other tissues (Table 2). LIP activity was low, but primarily isolated from proximal intestine preparations (Table 2). SDH activity was low and widely distributed.

#### Comparison of Plasma and Serum

There was minimal or no AMY, LIP, or SDH in the plasma or serum samples. There were notable differences between the plasma and serum samples for AST, ALT and GD in all lobsters. In all instances, serum values were higher than plasma at both time periods (Table 3). The amount of increase varied with the individual lobster and the enzyme. Changes in ALT were usually the most marked, whereas GD was relatively stable. Serum enzyme activity at 22 h tended to be less than at 5 h.

#### Screening Samples and Establishment of Reference Intervals

Enzyme activity collected 2 d after arrival was quite variable with some lobsters having AST and/or ALT activities up to 10 fold greater than the values obtained from the same animals only 4 d later (Table 4). A similar, but much less dramatic, finding occurred with LIP (Table 4).

Enzyme activity in the two data sets used for reference interval calculation had a nonGaussian distribution except for GD at 15°C. After removal of outliers, results from 25 of the 30 lobsters sampled after 6 d at 2°C, and 26 of the lobsters sampled after 4 d at 15°C were used to calculate the plasma reference intervals using percentiles (Table 4).

#### Gaffkemia Infection Trial

Animals received inocula of  $\sim 1.5 \times 10^6$  CFU/kg based on 48 h colony counts. A contaminant (*Acinetobacter* sp.) was detected in the inoculate suspension but not recovered from any lobsters. Bacteremia was detected on hemolymph culture in nine treatment group lobsters on postinoculation Day 2 and in all lobsters by Day 3. Four *A. viridans* colonies were detected in one control group lobster on Day 5 only. Bacteria were visible on DS in eight treatment group lobsters by Day 4 and in all by Day 5. A heavy growth of *A. viridans* was recovered from the hepatopancreas and antennal gland of all treatment group animals but no control group lobsters. Very low numbers of mixed bacteria were recovered from the antennal gland of nine, and from the hepatopancreas of three, control group lobsters.

THCs showed an initial increase Days 1–3 in treatment group lobsters followed by a decrease to  $\sim 2 \times 10^9$  cells/L by Day 5 in most (8/10) lobsters as seen in previous studies (Stewart et al. 1969, Stewart et al. 1983, Battison et al. 2004). A progressive decrease in THC ( $< 5 \times 10^9$  cells/L) was also noted in 5 of 10 control group lobsters. TP<sub>tr</sub> decreased by 10% to 15% of initial values by the end of the study in all, but one control (49%), lobsters.

For most lobsters, enzyme activities stayed within the previously established reference intervals (Fig. 1). There was a tendency for LIP to decrease over the course of the trial for both groups. A few animals (treatment and control groups) had minimal increases in ALT. Mild to moderate increases in GD were ob-

TABLE 2.

Activity of six enzymes\* in six tissues expressed as U/gram of total protein (biuret method) in tissue homogenate supernatants from four lobsters (*Homarus americanus*). Results presented as mean and (range) of values obtained.

	AMY	LIP	SDH	ALT	AST	GD
Heart	0 (0–0)	1 (0–1)	2 (2–2)	1425 (1311–1956)	2243 (1593–2970)	573 (358–602)
Antennal gland	0 (0–0)	0 (0–1)	3 (2–3)	246 (192–341)	562 (501–624)	16 (7–30)
Proximal intestine	0 (0–4)	3 (2–5)	1 (1–1)	518 (405–678)	314 (293–334)	67 (62–70)
Hepatopancreas	13 (7–19)	0 (0–1)	4 (2–4)	134 (5–342)	829 (706–1438)	6 (3–10)
Abdominal Muscle	0 (0–0)	0 (0–0)	1 (0–1)	157 (116–180)	449 (373–507)	32 (14–45)
Hemocyte lysate	0 (0–0)	1 (1–1)	0 (0–0)	323 (220–397)	101 (46–138)	52 (45–60)

\* Abbreviations: AMY, amylase; LIP, lipase; SDH, sorbitol dehydrogenase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GD, glutamate dehydrogenase.

served in 3 lobsters (2 treatment and 1 control). Elevations in SDH were limited to one treatment group lobster, which also had moderate increases in AST, ALT and GD and two control group lobsters—one of which had moderately elevated GD. Essentially no enzyme activity was detected in the TSB culture supernatant.

The main gross finding in the treatment group lobsters was the presence of multifocal to diffuse, white, milky, spots on the antennal gland. All of the control group lobsters showed variable degrees of pigmentation at the base of the gill filaments. Two treatment group lobsters had mild, focal, white discoloration of abdominal muscle segments (necrosis) near hemolymph collection sites.

Histological findings in the treatment group were consistent with gaffkemia (Johnson et al. 1981, Battison et al. 2004). Coccoid bacteria in tetrads were present in the hemolymph spaces of all tissues. Small hemocyte nodules, some with phagocytosed cocci, were noted in low to moderate numbers in most tissues examined.

There was moderate to marked, multifocal to diffuse, involvement of the fixed phagocytes in the connective tissue of the hepatopancreas. The cells were enlarged and vacuolated, containing large numbers of bacteria and often surrounded by hemocytic

nodules (Fig. 2). Deposition of a red-orange, hyaline material was noted in some nodules (Fig. 2). The antennal glands were similarly affected with multifocal to coalescing septic, variably sized, hemocytic nodules. Moderate, focal, myonecrosis was confirmed in one lobster.

All control group lobsters had a moderate to severe necrotizing, nonseptic, melanizing, branchitis. Large hemocyte aggregates were present in many gill filaments sometimes filling the vascular channels, similar to a thrombus. Adjacent tissue was uniformly lightly eosinophilic (necrotic) in some sections. Infectious agents were not identified. Neither *A. viridans* nor lesions typical of gaffkemia were found in the one control group lobster which had a single positive hemolymph culture.

## DISCUSSION

Clinical chemistry is an important diagnostic tool in human and veterinary medicine. The purpose of this investigation was to determine if this tool could also be applied to lobsters, and potentially other crustaceans. To interpret hemolymph enzyme activity, it was necessary to determine the tissue distribution of the enzymes in lobsters. This was important because enzymes that are tissue spe-

TABLE 3.

Activity of three enzymes\* (ALT, AST, GD) measured in plasma (processed immediately) and serum (allowed to clot for 5 and 22 h at 4°C) samples from four lobsters (*Homarus americanus*). Fold increase from plasma is indicated in parentheses.

	Plasma	Serum @ 5 h	Serum @ 22 h
ALT (U/L)	31	237 (7.6×)	106 (3.4×)
	18	286 (15.8×)	102 (5.6×)
	19	171 (9.0×)	167 (8.8×)
	15	399 (26.6×)	351 (23.4×)
AST (U/L)	38	185 (4.8×)	90 (2.3×)
	24	206 (8.5×)	85 (3.5×)
	16	111 (6.9×)	124 (7.7×)
	15	n/a**	164 (10.9×)
GD (U/L)	11	18 (1.6×)	15 (1.3×)
	10	18 (1.8×)	15 (1.5×)
	9	13 (1.4×)	16 (1.7×)
	19	24 (1.2×)	26 (1.3×)

\* Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; GD, glutamate dehydrogenase.

\*\* Not available.

TABLE 4.

Plasma enzyme\* activity in 30 lobsters (*Homarus americanus*) two days after arrival expressed as mean (and range) and reference intervals calculated after 6 d at 2–4°C and 4 d at 15°C.

	2 d @ 2–4°C (n = 27)***	Reference Interval** (2–4°C) (n = 25)****	Reference Interval** (15°C) (n = 26)*****
AMY (U/L)	0 (0–0)	0–0	0–0
LIP (U/L)	8 (5–20)	0–4	4–9
SDH (U/L)	0 (10–14)	0–19	0–0
ALT (U/L)	28 (12–300)	6–24	6–21
AST (U/L)	25 (8–319)	5–16	4–12
GD (U/L)	17 (7–31)	6–26	10–34

\* Abbreviations: AMY, amylase; LIP, lipase; SDH, sorbitol dehydrogenase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GD, glutamate dehydrogenase.

\*\* 5<sup>th</sup>–95<sup>th</sup> percentiles.

\*\*\* Three samples clotted, data not available.

\*\*\*\* Five outliers removed.

\*\*\*\*\* Four outliers removed.

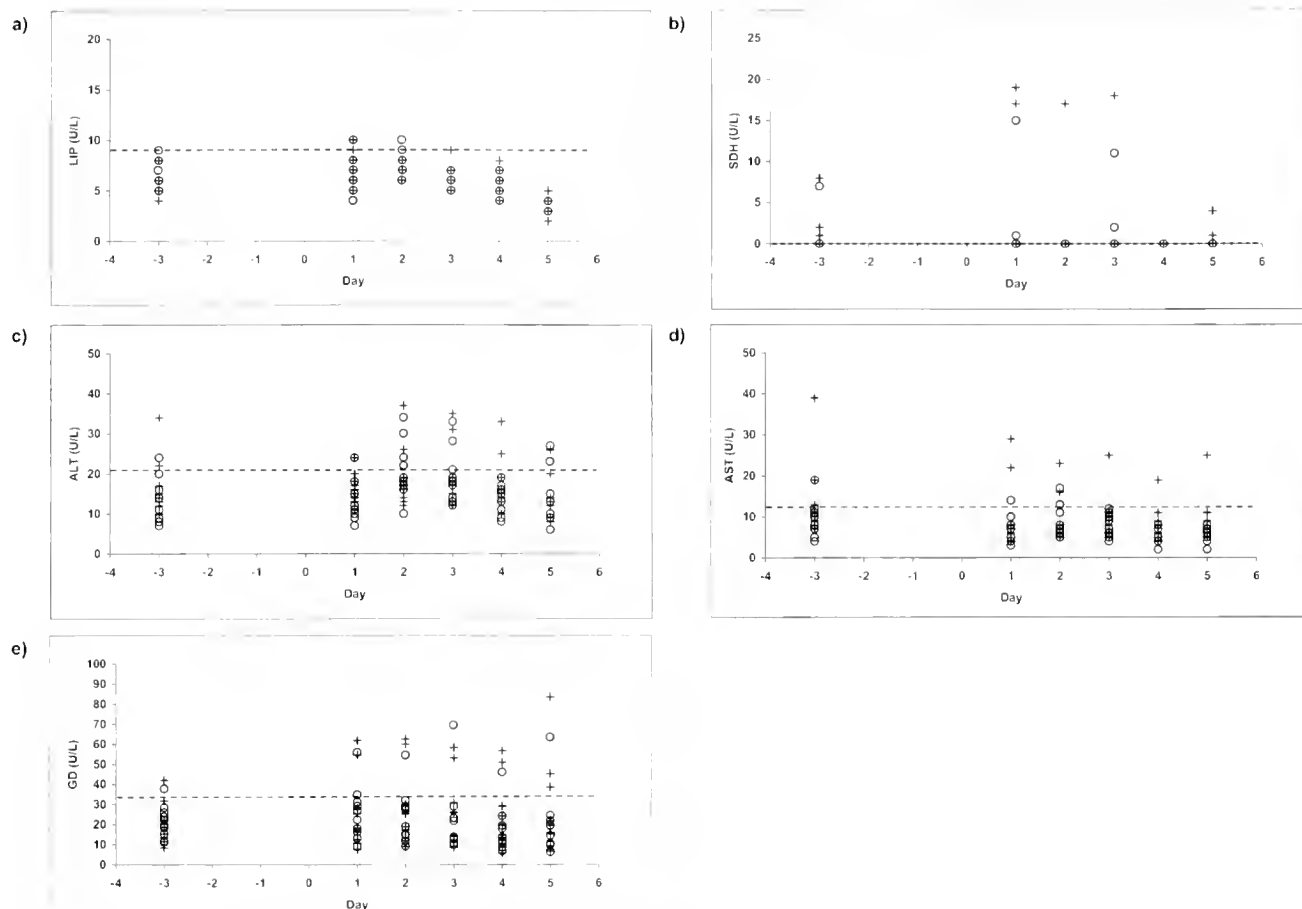


Figure 1. Plasma activity of: (a) lipase (LIP), (b) sorbitol dehydrogenase (SDH), (c) alanine aminotransferase (ALT), (d) aspartate aminotransferase (AST) and (e) glutamate dehydrogenase (GD) for lobsters (*Homarus americanus*) inoculated with *Aerococcus viridans* bacteria (+;  $n = 10$ ) or 3% sterile NaCl (○;  $n = 10$ ). Samples were collected prior (Day -3) to inoculation on Day 0 and daily during the trial (Days 1–5). The dashed line represents the upper limit of the reference interval for plasma activity for each enzyme.

cific in one species will not always be so in others (Duncan et al. 1994a).

Biochemistry panels in veterinary medicine may include enzymes of skeletal muscle (creatinine kinase (CK), AST, ALT), hepatocellular (AST, ALT, SDH and GD) and pancreatic (AMY, LIP) origin as a means to assess damage to, or dysfunction of, these tissues in addition to data on electrolyte and metabolite concentrations (Kramer & Hoffmann 1997, Moss & Henderson 1999). Enzymes present in the kidneys and intestine are not generally found in the blood in vertebrates (Kramer & Hoffmann 1997, Moss & Henderson 1999). Instead, when these tissues are damaged, enzymes are believed to be released into the urinary space or intestinal lumen rather than into the circulation (Kramer & Hoffmann 1997). A similar situation is expected for the antennal gland and intestinal tissues in the lobster. When injury to the heart is suspected in vertebrates, the results of more specialized testing (e.g., isoenzyme determination) are combined with other clinical information to differentiate cardiac from general muscle injury because the enzyme composition of the tissues is very similar (Moss & Henderson 1999).

Overall, tissue enzyme activity in lobsters in this study resembled the distribution seen in other animals (Moss & Henderson 1999), with some exceptions. The low activity of GD recovered from the hepatopancreas was surprising because this mitochondrial

enzyme is central to ammonia metabolism and is known to be present in crustaceans (Claybrook 1983). An inhibitor may be responsible. GD activity could be detected in the isolated mitochondrial fraction, but not in homogenates, of the hepatopancreas in the crayfish (*Orconectes limosus*) (Claybrook 1983). A similar effect could account for the results obtained in this study. Plasma from apparently healthy lobsters often contains moderate levels of GD—presumably of muscle and/or hepatopancreatic origin.

AST and ALT are commonly used as indicators of hepatocellular injury in vertebrate species (Duncan et al. 1994a, Moss & Henderson 1999). Skeletal muscle injury is usually indicated by increases in CK and AST, but may also be accompanied by increased ALT in severe disease (Valentine et al. 1990, Kramer & Hoffmann 1997, Moss & Henderson 1999). For this reason, it is useful to have at least one enzyme that is considered tissue specific. CK, as a muscle-specific enzyme, serves this purpose in vertebrates (Kramer & Hoffman 1997, Moss & Henderson 1999). The equivalent enzyme in lobsters is arginine kinase (AK) (Horney et al. 2001). Unfortunately, neither an AK assay kit nor the reagents necessary for modifying the CK assay were available at the time of this study. Consequently, plasma AST and ALT activity in lobsters should be considered to be of either hepatopancreas or muscle origin.

SDH can be used as a specific indicator of hepatocellular injury

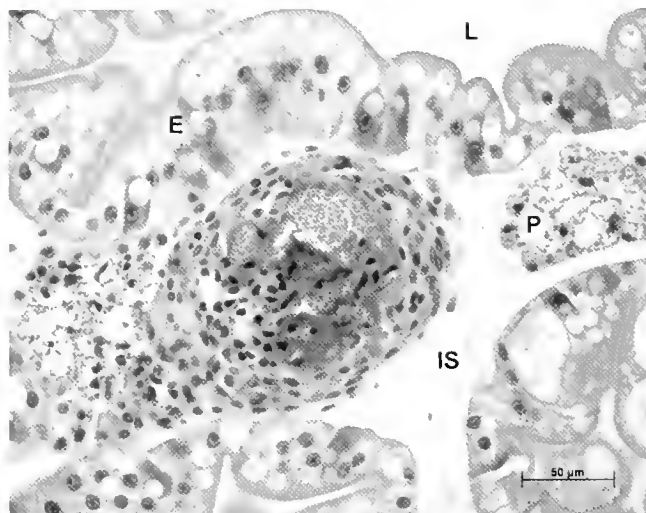


Figure 2. *Homarus americanus*, hepatopancreas. Hemocyte nodule in the intertubular space (IS). The nodule contains numerous cocci (*Aerococcus viridans*) in the center. The smaller group of cells at the upper right is a group of fixed phagocytes (P) with intracellular cocci. The hepatopancreatic epithelial cells (E) appear unaffected. The tubule lumen (L) is indicated.

(Duncan et al. 1994a, Moss & Henderson 1999). The relatively low SDH activities detected in multiple tissues in this study could indicate that either there is very little of this enzyme in these tissues, an inhibitor is present, or that the assay conditions (reaction temperature, substrate, etc.) were not optimal. Plasma SDH activity was often low or had poor repeatability. These findings suggest that SDH, under current assay conditions, may not be a useful enzyme for inclusion in lobster biochemistry panels. Further testing under different disease conditions will be required to confirm this.

Lipase is generally used as an indicator of pancreatic injury in vertebrates (Duncan et al. 1994b, Moss & Henderson 1999). Given the digestive function of the hepatopancreas, significant levels were expected in this organ as reported previously (Brockerhoff et al. 1967, Brockerhoff et al. 1970, Hoyle 1973, Biesiot & Capuzzo 1990). However, only relatively low activity was detected in the hepatopancreas with slightly higher activity in the proximal intestine in most lobsters in the current study. This discrepancy could be because of the different assay methodologies, or possibly an inhibitory substance. Differences in reporting enzyme activity among the studies make it impossible to compare the results. The presence of LIP activity in the intestinal preparations could indicate an additional location (i.e., enterocytes) for this enzyme or, incomplete removal of the lumen contents during sample preparation.

Detection of moderate levels of plasma LIP activity may be an indicator of recent feeding. Plasma LIP activities decreased while lobsters were held, fasted, at 2°C and increased when the animals were transferred to 15°C and fed. Refeeding after a period of starvation has been suggested as the cause of increased LIP activity in gastric fluid in *H. americanus* (Hoyle 1973). Plasma LIP activity decreased again in both groups as the trial progressed. Lobsters are reported to become inappetent shortly after infection with *A. viridans* (Stewart et al. 1972)—possibly a response to the inflammation associated with this disease. The branchitis may have caused a similar effect in the lobsters in the control group.

Unfortunately, food intake was not monitored closely enough in either group to confirm this hypothesis.

Amylase activity has been detected in hepatopancreas homogenates and gastric fluid in *H. americanus* (Wojtowicz & Brockerhoff 1972, Hoyle 1973, Biesiot & Capuzzo 1990). The hepatopancreas was the only tissue to consistently contain any significant AMY activity in this study. Because AMY activity was not detected in plasma samples from apparently healthy lobsters, this may indicate that plasma AMY will prove to be a sensitive and specific indicator of hepatopancreatic epithelial cell injury.

The reference intervals calculated for the two temperatures were quite similar, suggesting little effect of temperature. Whereas the intervals for male lobsters at 2°C to 4°C are likely an accurate reflection of lobsters in their natural surroundings, the intervals for 15°C may not be, because these lobsters were rapidly brought to this temperature under artificial conditions. Sampling lobsters that have gone through natural acclimation, with its attendant physiological adaptations, including possible induction of enzymes more suited to these higher temperatures, will be required to determine the validity of the current findings. Obtaining samples from a larger population of animals of both sexes at different molt stages from different fishing areas and during different seasons, is required to assess the possible effects of these variables.

Plasma is the preferred sample for measuring hemolymph enzyme activity. In the four animals tested, activities of ALT, AST and to a lesser degree, GD, were higher in serum than plasma. Activity of all three of these enzymes was found in the HLS. Thus, the serum activity is presumed to come from the hemocytes that lyse as part of the coagulation mechanism (Martin & Hose 1995). It is likely that the amount of enzyme contributed by the hemocytes will depend on both the total and differential hemocyte counts. A recent publication examined enzyme activity in hemolymph serum (Dove et al. 2005). It was noted that ALT and AST decreased in "warmer months," coincidental with absent hemolymph clotting. Poor clotting is frequently caused by hemocytopenia (Martin & Hose 1995)—the ALT and AST values obtained may have been an indirect indicator of the total and differential hemocyte counts rather than, or in addition to, release of these enzymes from tissues.

The benefits of using automated analyzers, with their internal standards and controls, include the relatively rapid determination of the activity of multiple enzymes in a small sample volume (~300 μL). Enzyme activity will be affected by reaction conditions such as substrate, temperature, and pH. For these reasons, reference intervals for enzyme activity are considered laboratory specific. In this study, all assays were performed at 37°C, the standard operating temperature of the automated chemistry analyzers and assay kits, although above the reported thermal tolerance (–1°C–30°C) of *H. americanus* (Lawton & Lavalli 1995). Previous work in homarid and panulirid lobsters and bivalves has detected enzyme activity when measured at 25°C and 37°C, with proteases of the spiny lobster demonstrating maximal activity at 50°C (Brockerhoff et al. 1970, Wojtowicz & Brockerhoff 1972, Hoyle 1973, Biesiot & Capuzzo 1990, Horney et al. 2001, Culloty et al. 2002, Celis-Guerrero et al. 2004, Gustafson et al. 2005).

Plasma enzyme activity appeared relatively stable when samples were refrigerated (2°C to 4°C) for up to 4 d or frozen at –20°C for 6 wk (results not presented). Stability studies were planned; unfortunately, only two samples with moderate enzyme activities were available for evaluation. The slight variations noted in these two samples may have been caused by the suboptimal

precision at these activity levels. Plasma AK had limited stability under different storage conditions and same day analysis was recommended (Horney et al. 2001).

Infection with *A. viridans* did not cause any consistent, specific, or clinically significant ( $>2\text{--}3\times$  increase above upper limit of the reference interval) changes in plasma enzyme activity in this study to allow early detection of the *A. viridans* infection. The hepatopancreas is a major site of inflammation in gaffkemia, yet there was little evidence of damage to this tissue on the biochemistry panels (i.e., clinically significant increases in AMY, LIP, ALT, or AST activity). This may have occurred because the inflammation was primarily located in the connective tissues with relatively little involvement of the hepatopancreatic tubule epithelium, the presumed location of these enzymes. Increases in plasma enzyme activity may have been observed if the trial had progressed until the lobsters died of the infection when the inflammatory lesions would have been more severe.

Clinical enzymology is dynamic with hemolymph enzyme activity reflecting current conditions. Five lobsters had very high AST and ALT activity 2 d after arrival—the highest enzyme activities observed in this study. The most likely origin of these enzymes would be muscle and/or the hepatopancreas. The values in all five lobsters had decreased significantly by the next sample collection only 4 d later, suggesting a transient insult, possibly related to trauma and/or hypoxia experienced during shipping. Three of these five lobsters (1 control and 2 treatment) showed progressive increases in GD accompanied by mild increases in AST and/or ALT during the gaffkemia trial. Whether the increase in GD was related to the previously elevated AST and ALT, or to some underlying process exacerbated by the *A. viridans* infection, remains speculative. There were no gross or histological lesions

unique to any of these five lobsters at necropsy to account for the enzyme changes.

## CONCLUSIONS

This study has identified the tissue distribution and established plasma reference intervals in *H. americanus* for ALT, AST, GD, AMY, LIP and SDH. This information can be used as a starting point to assess the usefulness of clinical enzymology for the diagnosis of disease in *H. americanus*. More information on the stability of these enzymes under different storage conditions, factors that can affect their circulating half life, possible induction and effects of the molt cycle on plasma activity, is needed. Plasma enzyme activities are most likely a tool to be used in research settings at present. Although plasma enzyme activity did not prove to be a sensitive test for detecting gaffkemia, with further investigation one or more enzymes may be identified that can be used to assess the general health of lobsters upon arrival at pounds (e.g., screening for the presence of underlying diseases) or detecting the effects of rough handling during transport, which might affect survivability under long-term holding conditions. This information could be useful when making marketing and management decisions.

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## COMPARISON OF EARLY SEASON VERSUS LATE SEASON TRAP SCHEDULES FOR HARVEST OF RED SWAMP CRAWFISH *PROCAMBARUS CLARKII* CULTURED IN EARTHEN PONDS WITHOUT PLANTED FORAGE

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**ABSTRACT** The effect of a redistribution of the majority of total trap harvest days (69) during April through October on yield of red swamp crawfish *Procambarus clarkii* cultured in earthen ponds without planted forage was evaluated in northeast Mississippi, USA (Latitude 33.45 and Longitude 88.82). The majority of trap days occurred from either April through June (55.1%), early harvest schedule, or July through October (62.3%), late harvest schedule. Twelve ponds (0.04–0.05 ha), 6 replicates per trap harvest strategy, that already contained established populations were provided a pelleted feed during 10 mo and trap harvested according to a common protocol except for the different monthly schedules. For the early and late harvest schedule treatments, mean total production (2448 and 2269 kg/ha), mean number harvested per ha (105,770 and 104,411/ha), and mean individual harvest weight (23.6 and 22.1 g) were not significantly different. These results suggest that the majority of trap harvesting in ponds without planted forage can occur after trap harvest in forage based culture ponds and natural habitats is usually terminated. When traditional supply is low, price and demand can be high in certain markets. This successful shift in harvest days introduces flexibility in management strategies that could be critical to the financial success of an enterprise based on production of crawfish in ponds without planted forage.

**KEY WORDS:** red swamp crawfish, *Procambarus clarkii*, crawfish aquaculture in deep earthen ponds

### INTRODUCTION

Previous research has been conducted on red swamp crawfish, *Procambarus clarkii*, cultured in earthen ponds without planted forage to evaluate feasibility (D'Abramo & Niquette 1991), initial stocking densities (D'Abramo et al. 2003), harvesting schedules (D'Abramo et al. 2003) and addition of substrate to culture ponds (D'Abramo et al. 2006). A summary of these results and the suggested management strategies are summarized in D'Abramo et al. (2002) and D'Abramo et al. (2004). Trap harvesting in traditional or forage-based crawfish production ponds in Louisiana commonly occurs from December through May. Ponds are usually drained in either May or June, and pond bottoms are then dried and planted with an agricultural crop such as rice or sorghum. Ponds can be drained as early as February or as late as August and this management decision is based on economic considerations. Extended harvests in forage-based ponds more likely occur at comparatively higher latitudes. The crawfish harvest season in Louisiana natural fisheries, centered in the Achafalyan Basin, can extend the availability of crawfish into June depending on the amount of rainfall, water level and subsequent boat accessibility within the basin (Huner 1997). Eversole (1988) evaluated two trap harvesting schedules in forage-based ponds in South Carolina and despite a significant reduction in forage present, similar production was achieved by utilizing a trap schedule that occurred from April to May or extended through June.

June through October is when crawfish are not typically available and price and demand can be high in certain markets. Utilization of this trapping schedule could provide a producer the ability to extend product availability and provide the opportunity to maximize profit by avoiding harvesting and marketing during periods of high supply and low price.

The objective of this experiment was to evaluate the effect of two trapping schedules with different temporal arrangements of

trap days on total production, the number of crawfish harvested per ha and the mean individual weight of harvested crawfish in earthen ponds without planted forage.

### MATERIALS AND METHODS

#### *Experimental Treatments and Ponds*

There were two experimental treatments, early and late season, referring to when the proportionately higher level of trapping occurred during a 7-mo period of April 1 to October 24. A total of 69 trap days occurred for each of the two treatments. For the early season treatment, 55.1% of the trap days occurred during April through June, whereas for the late season, 62.3% of the trap days occurred from July through October (Fig. 1). Each treatment was randomly assigned to six replicate ponds.

A total of 12 freshwater earthen ponds ranging from 0.04–0.053 ha in surface area and a mean depth of 1.2 m were used. The ponds were rectangular shape, between 1.5 and 1.8 m at the deepest point. Ponds were stocked 4 or 5 y before the initiation of this experiment and the resident population of crawfish originated from natural recruitment. All ponds had been in continuous production for 4 or 5 y and had been previously used for experiments investigating various management strategies for the production of red swamp crawfish without planted forage.

Vertically suspended within the water column and below the water surface of all the experimental ponds was a substrate consisting of orange, plastic safety barrier fencing (30.5 m length and 1.2 m height). The area of substrate in each pond was equivalent to 50% of the bottom surface area of the pond and was estimated by assuming it was a one-sided solid sheet (36.6 m<sup>2</sup>). The substrate was attached to 1.5 m steel reinforcement bars (four bars per 30.5 m length of substrate) with twine (D'Abramo et al. 2006) and had been in the ponds continuously for three years of growth, harvest and recruitment of crawfish populations associated with previous experiments.

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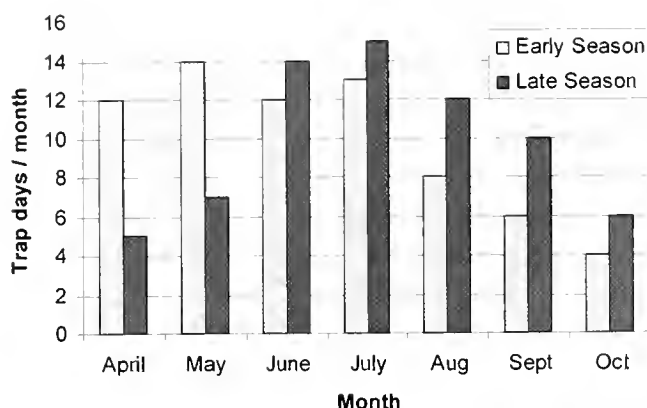


Figure 1. The number of trap days each month from April to October for the early and late season harvest experimental treatments.

### Feed

A 32% crude protein, sinking, formulated feed manufactured by pelletization (Producers Feed Company, Isola, MS) was added to the experimental crawfish ponds during the entire calendar year. The feed is considered to serve principally as an indirect organic fertilizer rather than a direct source of nutrition, serving to stimulate the rates of natural productivity in the ponds. The total amount of feed provided to each pond during the 12-mo period prior to and during the harvest of crawfish was 6,750 kg/ha. Monthly feeding rates were based on the estimated resident biomass of crawfish and water temperature (Table 1) and followed recommendations of D'Abramo et al. (2002).

### Harvesting

Three funnel (3.8 cm inner diameter entrance) pyramid traps constructed with hexagonal wire mesh (1.9 cm diameter openings) (Gulf Coast Wire Products, Kaplan, LA) were used and positioned along the perimeter of each pond at a density of 81/ha. The traps had necks that extended above the water surface and were longer (60 cm) than the necks of traditional pyramidal traps to accommodate the greater depth characteristic of ponds without planted forage. At the top of the necks, polyvinyl chloride (PVC) retaining rings (9 cm height) were located to prevent escape of crawfish

through the top of the trap. Each time a trap was baited, approximately 100 g of artificial bait (Gros Rouge Crawfish Bait, Cargill, Minneapolis, MN) was added.

Trap days were scheduled differently for each treatment to achieve the necessary number of trap days designated for each month (Fig. 1). Traps were harvested on Monday, Tuesday, Thursday and Friday; or Tuesday, Thursday and Friday; or Thursday and Friday or Friday only to accommodate the necessary number of trap days assigned per month. Soak times for the traps were either 24 or 48 h depending on the number of trap days per week. If the next scheduled trap day was more than 48 h in the future, traps were inverted and reset 24 h prior to the next scheduled harvest. Trapping protocol based on water temperature, as described by D'Abramo and Ohs (2003), was followed. When trapping occurred during warm water conditions ( $>19^{\circ}\text{C}$ ), bait was included in the traps. At temperatures between  $15^{\circ}\text{C}$  and  $19^{\circ}\text{C}$ , traps were not baited and allowed to soak so crawfish could passively move into the traps, possibly seeking refuge. For each harvest day, the total number and total weight of crawfish harvested from each pond were determined and recorded.

### Water Quality

Water temperature of the ponds was recorded twice daily, and dissolved oxygen was recorded twice daily except during June through September when it was measured three or more times per day. When the dissolved oxygen concentration was anticipated to decrease below 5 ppm, aeration was provided to the ponds by a 0.5 hp aerator (Aerolator Corp., Kansas City, MO). Additional, emergency aeration was provided by a tractor driven paddlewheel when the dissolved oxygen concentration decreased below 3 ppm. Every third day, from May through August, pH was measured and recorded at approximately 13:00. All ponds were treated with gypsum at approximately 181.6 kg/ha in June as a prophylactic treatment to minimize the potential for potentially lethal alkaline pHs to occur.

### Statistics

A one-way analysis of variance (ANOVA) was used to determine if differences existed between treatments for mean production (kg/ha), number of crawfish harvested (number/ha) and overall mean individual weight (g) (SAS 1988). Differences were considered significant at  $P \leq 0.05$ .

TABLE 1.

Monthly and annual number of days and amount of feed provided to each of the experimental ponds.

	kg/ha	% of Total	Days/Month
January	0	0	0
February	0	0	0
March	0	0	0
April	1744	20.5	30
May	1097	17.4	31
June	1041	16.5	30
July	788	12.5	31
August	506	8.1	31
September	450	7.1	30
October	506	8.1	31
November	422	6.7	30
December	197	3.1	15
Total	6750	100.0	259

### RESULTS

There were no significant differences between treatments in mean total production ( $P = 0.48$ ), mean number harvested per ha ( $P = 0.93$ ), or mean individual weight of harvested crawfish ( $P = 0.19$ ) (Table 2).

TABLE 2.

Mean total production, mean number harvested, and mean individual weight of crawfish harvested from experimental earthen ponds according to early and late season harvest treatments.

Treatment	Mean Total Production (kg/ha)	Mean Number Harvested (number/ha)	Mean Individual Wt. (g)
Early season	2448 $\pm$ 160	105,770 $\pm$ 9296	23.6 $\pm$ 0.69
Late season	2269 $\pm$ 185	104,411 $\pm$ 10,915	22.1 $\pm$ 0.83



The 20.3% increase in the number of trap days that occurred in June through October for the late season treatment resulted in a corresponding 16.5% increase in total production and a 15.3% increase in the number of crawfish harvested during this same period. Accordingly, by assigning more trap days later in the harvest season (June through October) the total amount of crawfish harvested increases by approximately 375 kg/ha. Monthly trap days, harvest yield (kg/ha) and number harvested/ha, expressed as a percentage of the total, are presented in Table 3.

### DISCUSSION

The results of this investigation provide insight into the true seasonality of crawfish production at the latitude where the study occurred. Obtaining this information is only possible through the use of a management practice that does not include planted forage. By utilizing the management strategies in the present experiment, it seems that production of 2200–2500 kg/ha/year can be perpetually achieved in properly managed 0.5 ha earthen ponds during a 7 mo period with 69 trap days, regardless of which of the present study's trapping schedules is followed. Recently initial attempts to incorporate this method of crawfish production into one 0.81 ha pond produced harvested annual yields slightly less (~2000 kg/ha) than what has been achieved in the experimental ponds. Farmers who use earthen ponds without planted forage do have the option of these two management strategies for harvest.

In the current study, only 20.3% of the trap days were shifted from April and May to June through October. Previous research has shown that trap days can extend over a 12-mo period during some years with a possible exception of January and February, typically the coldest months of the year (D'Abramo et al. 2003). Therefore, it may be possible to shift more trap days to later in the year with a corresponding reduction in trap days in March. Nonetheless, sufficient trapping effort should most likely be maintained during April and May when the resident biomass of crawfish is highest. This strategy of continued removal by harvest should reduce the possibility of decreased growth and production, which could occur if high population densities are allowed to persist for prolonged periods. In addition, an appropriate level of harvesting of the resident population probably contributes to reductions in cannibalism and emigration.

The maximum trapping effort that can be imposed without altering the rate of natural recruitment is not known. Mean indi-

vidual weight data collection over many years of experiments have shown that in a typical 0.05 ha research pond, a reduction in mean individual size does not occur until more than 100 trap days per year are extended over 10 mo (D'Abramo et al. 2003).

It may be possible to maintain the same trapping effort during periods of the highest population density and further increase the number of trap days to the later portion of the harvest season. This strategy may further increase total production and potential profit, whereas not reducing the resident population to a level whereby natural recruitment for harvest during the following year is adversely affected.

Wholesale prices for crawfish vary from month to month. Mean monthly wholesale prices in Louisiana are \$3.85 per kg in November and decrease to \$1.32 per kg in June (Romaine et al. 2005). In Louisiana, the lack of a cultured or captured product from July to October means a wholesale price does not exist. If available from July to October in Louisiana, the product may not likely command the November price of \$3.85 per kg because of lower demand caused by a large supply of other readily available seafood such as crabs and shrimp. The application of wholesale prices of crawfish in Louisiana to markets outside of Louisiana is difficult because 90% of the crawfish produced there are consumed locally (Romaine et al. 2005).

As part of the economic analysis in a previous publication on nonforage-based crawfish production systems (D'Abramo et al. 2002), a mean price of \$3.31 per kg was used for the entire year. This is likely a conservative estimate from July through October, but is a good price estimate throughout the entire year in markets outside of Louisiana. Another notable difference between Louisiana markets and markets outside the region is the marketing chain and the various steps involved from producer to consumer in Louisiana (Romaine et al. 2005). These multiple steps to reach the consumer spread revenue to businesses in the various steps whereas in other regions the producer may receive a higher price because they market directly to the consumer or to a single wholesaler prior to the product reaching the consumer. Similar or higher selling prices could also be obtained by capitalizing on a seasonally based sale of crawfish as fish bait.

In the present study, utilizing the late season trapping schedule and shifting 20.3% of trap days from April and May to June through October increased production during the same time period by 16.5%. The critical information from this study for a producer is that there is no significant difference in production between the two trap schedules. A late season trap schedule can extend availability of product to later in the year during periods when prices may be higher, depending on local demand and the marketing effort of the producer. This could increase annual gross receipts and the potential profit of a nonforage based earthen pond crawfish production operation.

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TABLE 3.

Monthly trap days, harvest weight (kg/ha), and number of crawfish harvested per ha expressed as a percentage of the total for each of the early and late season harvest treatments.

Month	Early Season days/mo %	Late Season days/mo %	Early Season kg/mo %	Late Season kg/mo %	Early Season num/mo %	Late Season num/mo %
April	17.4	7.2	9.2	3.9	9.0	4.2
May	20.3	10.1	20.2	8.9	20.7	10.3
June	17.4	20.3	22.9	29.7	25.2	31.9
July	18.8	21.7	20.6	21.9	22.1	22.8
Aug	11.6	17.5	10.0	13.7	9.6	12.8
Sept	8.7	14.5	9.3	11.7	7.5	9.7
Oct	5.8	8.7	7.8	10.2	5.9	8.3
Total	100	100	100	100	100	100

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## A PERSPECTIVE ON ECOLOGICAL CONSIDERATIONS IN CRAWFISH POND AQUACULTURE

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**ABSTRACT** The suite of resident, migrant, breeding, and wintering waterbirds depending on this region includes grebes, pelicans, cormorants, anhingas, wading birds, waterfowl, coots, rails, gallinules, shorebirds, gulls, and terns. All taxa now use the artificial freshwater wetland habitat provided by the ag wetlands. These taxa include local, regional, continental and hemispherical populations. Crawfish ponds are especially valuable cool season habitat for predaceous waterbirds because they provide shallow water systems rich in invertebrate and small vertebrate prey during the period from midautumn through midspring when most ricefields are drained. Numerous other bird species use riparian areas around ag wetlands either as residents or seasonal visitors. Over 280 bird species have been identified in and around crawfish-rice systems in Louisiana. Whereas birds are conspicuous species associated with ag wetlands, numerous other vertebrate species including mammals, reptiles and turtles use the habitat. In addition, fishes in waterways receiving pond effluents receive significant food resources when water is released from the ponds.

**KEY WORDS:** crawfish, aquaculture, ecological services, ag wetland habitat

### INTRODUCTION

Freshwater crawfish, *Procambarus* spp., are cultivated in shallow-water impoundments by establishing self-perpetuating populations in permanent culture systems or mature crawfish in rotational culture systems (McClain & Romaine 2004). Most crawfish culture is located in Louisiana where 52,000 ha are devoted to crawfish production (LCES 2004). Most of the shallow-water impoundments are used to cultivate rice in warm months and are reflooded for crawfish culture in the cool months following rice harvest. These so-called agricultural (ag) wetlands are managed in such a way to follow the region's natural wetland cycle with alternate wet dry cycles. Crawfish mature in 4–6 mo and retreat to burrows during the heat of the summer when natural wetlands are either dry or water is too hot for survival and growth of the crawfish. The two important species are the red swamp crawfish, *Procambarus clarkii* and the white river crawfish, *Procambarus zonangulus*, whose basic biology and size are similar. See McClain and Romaine (2004), Huner (2002a) and Avery and Lorio (1999) for information about basic crawfish biology and fisheries and aquaculture management considerations.

Because crawfish are cultivated by simulating natural hydrological cycles in what amounts to be seasonally flooded wetlands, it should not be surprising that crawfish ponds provide ancillary ecological services (Huner 2002b). Furthermore, crawfish culture, as practiced with *Procambarus* spp. is probably the most sustainable form of pond aquaculture practiced in North America. That is, agricultural waste, rice stubble serves as the base of the food web that generates substantial crops of crawfish with minimal environmental impacts while providing economic benefits to communities (Huner 2002b, Caffey et al. 1996). This brief review discusses the various ecological services, foremost being waterbird habitat, provided by the agricultural community through cultivation of crawfish in shallow water, earthen ponds.

### WATERBIRD HABITAT

Huner et al. (2002) discuss at length the management of agricultural wetlands in the coastal regions of Louisiana, which ultimately benefit resident and migrating waterbirds. Over 280 bird

species including at least 75 waterbird species have been documented utilizing agricultural wetlands and adjacent riparian areas. This discussion is appropriate, however, for the South in general. Rice and crawfish farming operations are the main agricultural wetlands found in Louisiana. The following are water management practices associated with permanent and rotational crawfish ponds:

#### Permanent Crawfish Ponds

These systems produce crawfish on a continuing basis. When rice is planted as a forage base for crawfish in permanent ponds, grain is usually not harvested. Where rice is harvested, such systems are considered to be a type of rotational management—see later. Alternatives to rice as forage bases most commonly encountered include sorghum-sudan grass hybrid or volunteer vegetation including perennial forbs such as alligator weed, *Alternanthera philoxeroides*, smartweeds, *Polygonum* spp., duck potatoes, *Sagittaria* spp. and annual wetland grasses and sedges. These ponds are usually “open” in the sense that they are not wooded. However, there is some acreage associated with impounded forested wetlands (swamps). These so-called swamp ponds are usually very large compared with most ponds, regardless of type, that rarely exceed 20 ha in size. They typically have very poor crawfish production compared with all other crawfish pond systems.

#### Rotational Crawfish Ponds

In general, these systems produce crawfish in alternate years. However, some farmers harvest rice and crawfish from the same ponds year after year and are rarely restocked with crawfish after the first crawfish crop is harvested. In most rotational ponds, crawfish are stocked after the planting of rice and the establishment of a shallow “flood” of water once the rice has developed permanent leaves. Rice is managed for grain production and harvested. After rice harvest, the ponds are refilled in the fall and managed for crawfish production into the following spring. Once crawfish harvesting ceases, farmers may drain the fields and plant a crop such as soybeans or leave the fields fallow until rice is planted the following spring. Crawfish are then stocked and the cycle is repeated. If a crop like soybeans is not planted, farmers often choose to not drain fields until the following autumn to control weeds. They do not add water to the fields and water is lost to evaporation and percolation with rate being related to rainfall patterns. (See

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McClain & Romaine 2004, McClain 2005 and Romaine 2005 for more information about management of permanent and rotational crawfish pond systems.)

#### *Bird Feeding Niches and Behaviors*

The presence of water in the crawfish ponds provides feeding habitat and loafing habitat for all types of waterbirds including waterfowl, grebes, pelicans, cormorants, wading birds, shorebirds, coots, rails, gulls and terns. Furthermore, adjacent riparian areas and nearby wetlands attract numerous nonwaterbird species (Huner & Musumecche 1999, Musumecche et al. 2002). Each suite of birds has a particular feeding niche. Waterfowl and coots feed largely on rice left in the fields intentionally (permanent ponds) or as a consequence of harvesting processes (rotational ponds). They also benefit from seeds, green plant materials and tubers associated with volunteer vegetation especially where that is the crawfish forage base. Ducks (and coots) will also prey on zooplankton "blooms" concentrated along shorelines by winds especially in the spring and benthic invertebrates including crawfish when animal protein is required for egg formation. Wading birds and rails feed along pond margins when water is deep or throughout shallow areas present as ponds are filled or drained or because of untended water loss from evaporation or seepage. Hovering birds such as gulls and terns take prey from the water surface. These birds are especially adept at gleaning molting crawfish from emergent vegetation where they concentrate during daylight hours. Soft crawfish have very low mineral content and, being soft, are very easy to eat. Diving birds such as grebes and cormorants feed within the water column often in water so shallow that they cannot submerge. Pelicans, specifically American White Pelicans, form feeding lines and drive prey, including crawfish, toward shores where they are concentrated and easily scooped up. Shorebirds feed wherever shallow water is available—see later.

With the exception of shorebirds, all other species use crawfish traps that extend above the surface for resting places. Cormorants and wading birds are often seen to remove cut fish from traps when it is used for bait. Cormorants, wading birds and gulls regularly take crawfish that climb to the water surface inside and outside of traps.

There is no set time that ponds are drained and flooded. Because target flooding and draining dates are midOctober and mid-June (Avery & Lorio 1999), management considerations generate wide windows of flooding dates from midsummer to midfall and draining dates from early spring to late summer. Food organisms are most abundant when ponds are drained. Draining can take place as early as February or March even if the crawfish crop is too poor to justify harvesting, but, even though the crops are not worth harvesting, prey concentrates to desirable levels as water levels drop generating excellent feeding opportunities for carnivorous birds. In rotational rice-crawfish ponds, draining may be delayed until midfall to control weed growth prior to preparing soils for planting spring rice crops. The moist soils that are ultimately exposed are of special significance to shorebirds including wintering species or migrants. Such soils are sources of high densities of invertebrate prey especially insect larvae, crustaceans and annelid worms that provide the mainstay of shorebird diets.

Table 1 provides general information on the seasonal utilization of crawfish pond systems by waterbirds. See Huner et al. (2002) for more details.

In the case of rotational crawfish ponds, a practice that is es-

TABLE 1.

Seasonal abundance of major waterbird taxa in crawfish pond systems in southern Louisiana (USA).

Taxon	Season			
	SP	SU	F	W
Ducks	C	R	C	C
Grebes	R	U	U	R
Pelicans	—	—	—	R
Anhingas	R	R	R	—
Cormorants	U	C	C	U
Bitterns	R	R	R	R
Heron/egrets	C	C	A	C
Night-herons	U	C	C	U
White ibises	A	C	A	A
Dark ibises	A	C	A	A
Spoonbills	U	U	C	U
Storks	—	U	U	—
Coots	U	X	—	C
Shorebirds	C	U	A	U
Gulls	C	U	U	A
Terns	U	R	U	U

Legend. Seasons: SP = spring, March to May; SU = summer, June to July; F = fall, August–October; and W = winter, November to February. Seasonal abundance: A = abundant, widespread and easily found in proper habitat in large numbers; C = common, widespread and easily found in proper habitat, but generally not in large numbers; U = uncommon, widespread and present, but in low numbers at proper season; R = rare, found infrequently at proper season, but a general pattern of occurrence is evident.

pecially important to waterbirds involves leaving water in the pond once crawfish harvesting ceases. Water is not drained but allowed to evaporate. Because the bottoms of most of these ponds have been leveled carefully with laser technology, there is very little slope and variation in depth. Water remains across most of the pond bottom until it finally dries or it is drained to permit tillage for an agronomic crop the following spring. The presence of water reduces problems with terrestrial weeds when pond bottoms are tilled. In practice, water depths of 2.5–5.0 cm generate ideal habitat for shorebirds migrating through the area during the "fall" migration beginning in July and ending the following October. Likewise, large concentrations of post nesting wading birds frequent these sites and good numbers of early fall waterfowl such as Blue-winged Teal, Northern Shoveler and Northern Pintail are attracted to this shallow water/moist soil habitat.

#### *Wetland Habitat Considerations*

Rice and/or crawfish are cultivated in over 300,000 ha of shallow earthen impoundments within 160 km of the Gulf of Mexico from the central Texas coast eastward to southeastern Louisiana. The region includes the Gulf Coastal Plain and the Lower Mississippi River Valley. Annual loss of 4,475 ha of coastal wetlands over the past 50 y has significantly reduced desirable natural freshwater habitat in the region (Coreil 1993, Raynie & Beasley 2000).

The significance of agricultural wetlands as waterbird habitat becomes very clear when one notes that over 600,000 ha of adjacent coastal wetlands have disappeared since the 1950s (Coreil 1993, Raynie & Beasley 2000). What was once ideal waterbird habitat has become open water with no chance that it will be

restored in the foreseeable future. Therefore, "artificial" wetland habitat has replaced those lost wetlands.

#### *Landscape Considerations*

Crawfish are cultivated mainly in two distinct geographical regions in Louisiana—coastal prairie areas with light, loessal soils and floodplain areas with heavy, alluvial soils (Huner 2004a, 2004b). An impervious "hard pan" develops in cultivated prairie areas under several centimeters of topsoil and holds the water permitting the cultivation of rice and crawfish. The heavy clays in floodplain areas naturally hold water tenaciously and permit rice and crawfish culture. Interestingly, much of the area where crawfish is cultivated in floodplains was very low, flood prone, marginal land cleared in the 1960s to permit cultivation of soybeans. Within 5–10 y soybean cultivation became unprofitable and, at least in southern Louisiana, such fields surrounded by levees for flood control, were converted to crawfish ponds.

During the drought period of 1999 to 2001, there was a very real reduction in crawfish yields in the prairie regions of Louisiana, whereas crawfish yields in alluvial regions did not suffer as much (Huner 2004a, 2004b). To be sure, Icon, a systemic rice pesticide, was associated with considerable loss of crawfish in prairie regions. However, scrutiny of crawfish production from alluvial areas, especially where treated rice was not used, suggests strongly that drought resulted in a much greater reduction of crawfish production in the lighter prairie soils than in alluvial soils. Therefore, it is apparent that farmers need to manage soil moisture much more carefully in the prairie soils than the alluvial soils. This, then, benefits waterbirds as there is more moist soil/shallow water habitat available to them.

#### *Threats to Waterbird Habitat Provided by Ag Wetlands*

Farmers will cultivate rice and/or crawfish only as long as it is economically feasible to do so (Huner et al. 2002, Westra et al. 2005). Rice farming would not exist today were it not for farm subsidies. Crawfish was once considered to be a providential "cash" crop. Today, it is a principal source of income in a very troubled agricultural economy (McClain 2005). If crawfish, for whatever reason, fails to sustain the rice component of an integrated rice-crawfish enterprise then the enterprise will close and the wetland habitat will not be sustained. Farmers have been evaluating sugar cane as an alternative to rice in southwestern Louisiana. Sugar cane must be kept reasonably dry so cane fields lose shallow water/moist soil functions when a rice crop is replaced with a sugar cane crop.

In the case of absentee landowners, farmers leasing rice fields cease to farm rice if the practice based on rice values and lease costs exceed income. Fields are abandoned and are quickly overgrown by exotic tallow trees that form thick, impassable stands of trees in less than 5 y. The costs of clearing such trees are such that such properties are unsalvageable in the current agricultural economy.

Farmers are generally of the opinion that predaceous waterbirds negatively impact their crawfish crops. This issue is discussed at length by Huner et al. (2002). In the absence of replicated, controlled predation studies, ancillary observations suggest that birds such as wading birds, gulls, terns and pelicans probably do not negatively impact crawfish production directly when farmers practice recommended management procedures. However, larger birds using crawfish traps as perches do dislodge them causing loss of

bait and catch and, in that case, do have a negative impact on the crawfish operation. Waterbirds largely considered to be nonpredaceous, such as geese and coots, can adversely impact crawfish production indirectly by destroying emergent vegetation, especially rice stubble. Aside from providing fuel for the detrital system, the emergent vegetation serves as critical substrate for crawfish dispersal, provides access to the surface and atmospheric oxygen when dissolved oxygen levels are very low and cover from the myriad of predators, including birds, associated with all wetlands, natural or artificial, in the region.

Use of water, especially ground water, by farmers for irrigation of all crops is being carefully scrutinized as demands for water escalate for industrial and residential activities (Lutz et al. 2004, Huner et al. 2002, Lovelace 1994). In 2000 (DOTD 2002), Louisiana "rice" farmers used approximately 42% of the ground water used in the state. Increases in the cost of "energy" force farmers to be much more guarded in their use of water, regardless of source, because they must pay for the energy required to pump the water into pond systems. However, farmers will continue to use a very high percentage of the water consumed in Louisiana.

If resource managers do not recognize the critical wetland habitat created by rice and crawfish farmers, they could make water allocations that negatively impact waterbird populations with staggering implications especially when hemispherical shorebird populations are taken into account.

#### **OTHER ECOLOGICAL/SOCIETAL SERVICES**

All impounded agricultural areas hold water for varying lengths of time following rainfall events. Water levels in rice and crawfish systems are rarely held at drain level so all units have some freeboard that equates to water storage capacity (Huner 2002b). However, because drains cannot accommodate the volume of water falling into ponds during major rainfall events, ponds store the water for varying periods of time until the drains can discharge water temporarily stored in the ponds. Even empty ponds hold water until such time as it can drain through the openings cut in levees. As a result, rice fields and crawfish ponds hold water and reduce the impact of floods in lower reaches of receiving bayous, streams, and rivers, a definite benefit to homes and businesses. Furthermore, even when such locations drain completely, soils remain damp for various lengths of time creating landscape level moist soil vistas. There are at least 240,000 ha of land used for rice production and surrounded by levees in Louisiana during nonrice production seasons (LCES 2004). No more than 20% to 25% of this land is flooded for crawfish production then.

Water is discharged from crawfish ponds for aeration purposes, to eliminate excess rainwater, and to drain ponds during the spring and summer (Lutz et al. 2004, Huner et al. 2002, Lovelace 1994). Concerns about discharge of pollutants, especially nutrients and suspended solids have been addressed by the development of Best Management Practices (BMPs) (Lutz et al. 2004). In general, water quality is not generally so poor as to cause excessive concern as long as the BMPs are followed (Parr et al. 2004).

The waters discharged from crawfish ponds benefit the receiving waters in 2 ways (Huner 2002b). First, water is often discharged during very dry periods in the mid-late fall and late spring/early summer when receiving waters exhibit seasonally poor water quality. Therefore, the increase in volume and movement of water generated by discharges actually, it can be argued, improve water quality. Second, fish food organisms are discharged

with effluent providing feeding opportunities for fish and other predaceous aquatic organisms comparable, it can also be argued, to discharges from natural wetlands.

### CONCLUSIONS

Crawfish culture, as practiced in the southern USA, represents a form of highly sustainable aquaculture that has very real benefits to wildlife and fisheries resources. Crawfish impoundments are artificial wetlands, comparable in many ways to natural wetlands in the region. Resources managers should encourage crawfish aquaculture wherever it is economically feasible and seriously consider managing wetlands on state and national wildlife management properties in a similar manner.

Incorporation of crawfish management into government-sponsored land conservation programs should encourage landown-

ers to sustain standing water habitat outside of program mandated fill/drain requirements. Farmers could adjust the times when their ponds are filled or drained to maximize benefits to many species, especially migrating shorebirds. The issue of possible damage done to crawfish crops by predaceous birds remains unresolved but, if such birds are proven to be deleterious, compensation to encourage farmers to continue to cultivate crawfish would certainly be justified on the basis of the ecological value of their ponds to wildlife.

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## EMBRYO DEVELOPMENT AND MORPHOMETRY IN THE BLUE KING CRAB *PARALITHODES PLATYPUS* STUDIED BY USING IMAGE AND CLUSTER ANALYSIS

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**ABSTRACT** In this paper the embryonic development of laboratory-reared blue king crab, *Paralithodes platypus*, from the Pribilof Islands in the eastern Bering Sea is described. Developing embryos were removed from a female crab at various intervals, digitally photographed under a compound microscope and analyzed using Image-Pro Plus. Nine morphometric parameters were used, including seven measurements (total area, yolk area, embryo length and width, average diameter, eye length and width) and two calculated indices (percent yolk and elongation). First cell division was not apparent until day 4, after which divisions occurred daily until the blastopore appeared at day 28. A “V”-shaped embryo became apparent on day 114, followed by rapid appendage development. The eyes became pigmented by day 192. Hatching occurred from day 381 to day 409, and required at least 33 d to complete. Embryo area declined from an initial value of 0.95 mm<sup>2</sup> on day 1–0.83 mm<sup>2</sup> on day 72 and then increased to 1.28 mm<sup>2</sup> on day 388. Growth of all characters reached a plateau between days 240 and 353, and then increased rapidly until the middle of hatching (day 390). Visual examination was better at defining early changes, but cluster analysis of morphometric measurements was a better technique for defining middle and later stages. Both techniques resulted in an optimum selection of 12 developmental stages. Embryonic development has been described for few decapod crustaceans, and no standard exists for defining developmental stages. Multivariate analysis of morphometric measurements may lead to improved understanding of crustacean embryogenesis, allow standardization of staging and enable studies of environmental influence on development. The technique also has applications in the aquaculture industry.

**KEY WORDS:** king crab, development, hatching, incubation, embryo, morphometry, image analysis

### INTRODUCTION

Studies of crustacean population fluctuations must include techniques for assessing environmental impacts such as climate change on reproduction, including embryonic development rates, diapause and irregular embryogenesis. Previous studies of embryo development in decapods have relied on traditional visual techniques to define developmental stages (Moriyasu & Lanteigne 1998, Yamaguchi 2001). However, the subjective nature of these methods leads to high variability because of the lack of standardized techniques or equipment, and such studies can rarely be applied to other species. Recent developments in digital imaging equipment and software have made it possible to improve the quality and reliability of morphological assessments in human and veterinary medicine. Image-analysis techniques have been used successfully to assess sperm morphology and quality in humans (Verstegen et al. 2002), horses (Hidalgo et al. 2005), fish (Gage et al. 2002), and marine mammals (Kita et al. 2001), and these techniques are easily adapted to studies of embryos as well. Crustaceans are particularly suitable research subjects because of their external and easily accessible embryos. Morphometry of embryos has been used to describe developmental stages in the freshwater prawn *Macrobrachium borellii* (Lavarias et al. 2002).

King crabs are large anomurans that are commercially exploited in many parts of the world. Commercially valuable species include red and blue king crab *Paralithodes camtschaticus* (Tilesius, 1815) and *P. platypus* Brandt, 1850, respectively, golden king crab *Lithodes acquispinus* Benedict, 1894, scarlet king crab *L. couesi* Benedict, 1894, European king crab *L. maja*, southern king crab *L. santolla* and others. Despite their value, embryonic development has only been studied for red king crab in Japan (Nakanishi 1987). There is no standardized scheme for describing devel-

opmental stages for king crab or any other large decapod crustacean.

Blue king crab (BKC) have historically supported lucrative fisheries in the eastern Bering Sea (EBS) at St. Matthew Island and near the Pribilof Islands (St. Paul and St. George). In 1999, both populations declined drastically, and their fisheries were closed, leading to renewed interest in research on their biology. Blue king crab have a 2-y reproductive cycle; in the first year of this cycle (the spawning year), female crabs molt, extrude eggs, mate and carry the developing fertilized embryos for approximately 1 y (Somerton & MacIntosh 1983, Jensen et al. 1985, Somerton & MacIntosh 1985, Jensen & Armstrong 1989). During the second (or hatching) year, larvae are released, but the crabs do not molt or mate again (Stevens in press), unlike female red king crabs that hatch, molt, mate and extrude annually (Stevens & Swiney, in press).

This research was undertaken as part of a larger study on the early life history of BKC, including development of cultivation techniques to ensure a supply of small crab for future research (Stevens et al., in press). This article describes the embryonic development of BKC during cultivation in the laboratory and methods to standardize definitions of developmental stages using visual as well as morphometric characteristics.

### MATERIALS AND METHODS

Blue king crabs were captured by trawl about 20 miles northeast of St. Paul Island, in the eastern Bering Sea, during October 2003 (for dates and locations see Stevens in press). Crabs were kept in recirculating seawater aboard ship for several days until returning to Dutch Harbor, Alaska. They were then packed in insulated coolers between layers of wet burlap, kept chilled with frozen ice packs and shipped by air to Kodiak, Alaska. Upon arriving in Kodiak, crabs were placed in a 2500-L tank containing chilled (4°C) seawater. Most female crabs were new-shelled and ovigerous, but two female crabs had old shells and empty egg cases, evidence that they had released larvae the previous spring.

When one of the old-shell crabs subsequently molted, it was

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Reference to trade names does not constitute endorsement by the National Marine Fisheries Service.

placed in a tank with a male crab and observed daily during grasping until new clutches of eggs were extruded and the female was released. Examination of embryos began 1 d after fertilization (day 1). A small cluster of 50–100 eggs was removed every other day for the first 3 wk and at 2–3 wk intervals thereafter. Half of the eggs from each sample were examined live, and the remaining eggs were stained for 5–15 min in Bouin's solution prior to examination. Eggs were placed on a glass slide in 1 ml of filtered seawater and examined under a compound microscope at  $\times 50$ -magnification using reflected light (darkfield background) from a fiber-optic source. Digital photographs of live embryos were taken with a 2-megapixel digital camera (Diagnostic Instruments Spot Insight camera) and analyzed using Image-Pro Plus, version 4.5. From days 3–12, individual photographs were taken of 3–5 embryos, but after day 12, 10–12 embryos were photographed on each sampling date. Embryos were only photographed if they were rotated at 90° to the sagittal plane. The image analysis system was calibrated using digital photographs of a stage micrometer set to the height of the midplane of crab embryos; the mean value (pixels  $\cdot$  mm<sup>-1</sup>) from three digitized images was used. Measurements were made by first outlining the embryo on the computer screen using the computer mouse; if the inner and outer embryo membrane were clearly separated (as was often the case during the first 90 d of development), then the inner membrane was outlined. If the embryo outline was clearly defined and free of background clutter, then the automatic tracing option was used. The outline was then captured using a smoothing value of 5 (on a scale of 1–9). For the first 3 mo, digital measurements collected for each embryo included area (A), maximum diameter (L), minimum diameter (W) and mean diameter (calculated from 180 measurements taken at 2° intervals around the perimeter). After the embryo became apparent at day 114, the area of the yolk mass was determined, and the percentage of apparent cross-sectional area occupied by yolk (PAY) was calculated. After day 192, 10 additional embryos were photographed where the eyespot was rotated to the top central position, and L and W of the pigmented eyespots were measured. In addition to the measured parameters, three metrics of embryo shape were calculated, including ellipticity (L/W), elongation ( $[L - W]/[L + W]$ ), and circularity ( $\pi LW/4A$ ) (Hidalgo et al. 2005). Measurements were output directly to an Excel spreadsheet for analysis.

When hatching began, the female crab was placed into a 70-L plastic tub fitted with a bulkhead fitting and drain on the lowest portion of the sidewall. Tubs received flowing sand-filtered seawater at a rate of 4–5 L  $\cdot$  min<sup>-1</sup> at ambient temperature ( $4.8 \pm 0.4^\circ\text{C}$ ) during the hatching period. Larvae exiting the drain passed up through an exterior standpipe and into a fine mesh net. The net was removed daily and larval volume measured to the nearest 0.5 mL in a graduated cylinder. Mean hatching date was determined as the weighted average of larval production over time, that is, by multiplying the daily volume of hatched larvae by day-of-the-year, summing the products over time and dividing by total volume of larvae released.

#### Stage Descriptions

Stages of embryonic development were defined using two different methods. One was the traditional method using developmental changes that are visually observable in the stained or unstained embryos. However, no standard criteria exist by which to define stage endpoints, so stage definitions tend to be subjective.

As a general guide, reference was made to descriptions of embryonic development in snow crabs *Chionoecetes opilio* (Moriyasu & Lanteigne 1998) and red king crab (Nakanishi 1987).

In the attempt to develop a more objective morphometrically-based method of classifying embryonic stages, cluster analysis of embryo morphometry was used as a second technique (Ludwig & Reynolds 1988) using SAS PROC CLUSTER. By grouping together samples (dates) with similar characteristics, the clustering technique should identify groups of dates (which may represent periods of development if they are sequential) during which the embryo metrics are most similar to each other and thus represent stages of development with little change. Different stages of development should be grouped into different clusters. Sampling units were defined as dates (with individual embryos as replicates), and the method used was average Euclidean distance. However, the actual sample dates were not used as input data because they would have influenced the resulting order of clusters, whereas the goal of this analysis is to determine the stage of development in randomly sampled (wild) crabs whose fertilization dates are unknown. All 11 measured and calculated metric parameters were used in the initial cluster analysis except yolk area (which was only used for calculating percent area of yolk, PAY). Subsequent analyses were made by removing calculated indices until the clusters were aligned in best chronological order. The number of clusters defined is somewhat arbitrary, with a maximum up to the total number of samples. However, selection of an appropriate number can be guided by looking for peaks in the pseudo-*F* statistic, and valleys in the pseudo-*r*<sup>2</sup> statistic (SAS 2004). Values of morphometric parameters are given in the text as Mean  $\pm$  1 SD.

The utility of this method for classifying the stages of eggs from wild crabs with unknown developmental histories depends on the conditions under which it is applied. As a test, sampled data from three different crabs with embryos of different known ages were included and classified along with data from crab #1. Each test sample consisted of measurements from 10 embryos, and each crab had been held at different temperatures, as follows: Crab #2, 2°C, 167 d; Crab #3, 4°C, 192 d; Crab #4, 6°C, 223 d.

## RESULTS

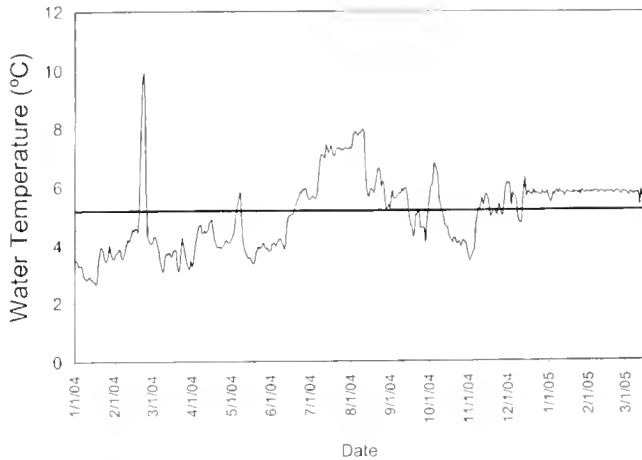
#### Embryo Development

Female crab #1 molted on January 17, 2004, was grasped and mated by the male the next day (18 January) and was observed to have extruded eggs on the next day, 19 January 2004, which was designated as day 0. Water temperature increased gradually from 4°C to 6°C during the study; mean temperature was  $5.2^\circ\text{C} \pm 1.2^\circ\text{C}$  during the incubation period (January 19, 2004 to March 3, 2005) (Fig. 1). The following description of development is organized according to the 12 stages defined by morphometric cluster analysis (see later).

#### Stage 1 (Cleavage)

During this period dividing cells were easily distinguishable prior to blastodisc formation, and little change in morphometry occurs. Eggs were initially lavender colored; first divisions occurred on day 4, but true 2-cell stages were not observed. By day 7, all embryos were multicellular, including 4-cell stage and 8-cell stages, and a few with 16 cells (Fig. 2A, a). On day 9, most embryos were at the 32-cell stage and one was 64-cells. Nuclei were apparent as diffuse light-colored spots in the center of each





**Figure 1.** Water temperature during embryonic development of blue king crab (*Paralithodes platypus*). Mean temperature (heavy line) was  $5.2 \pm 1.2$  C.

cell. Embryos were separated from the outer egg membrane, which had increased slightly in size. Cell numbers continued to double at 2-d intervals, to 64 on day 10, 128 on day 12, 256 on day 14, etc. By day 19, yolk had broken up into small irregular globules and cell borders were no longer distinct, although nuclei were apparent on eggs preserved in Bouin's. This period corresponds to visual stages V1-V2 in Table 1.

#### *Stage 2 (Blastula-Gastrula)*

Individual cells and structures are not visible. The blastodisc became apparent on day 28 and the blastopore was visible on some eggs (Fig. 2B, b). Embryos continued to decrease in size until day 72, but developmental changes were not discernible. Corresponds to V3-V5.

#### *Stage 3 (V-embryo to Nauplius)*

During this period embryonic lobes are becoming visible and are increasing in size. On day 114, the V-shaped embryo became distinct from the yolk in stained eggs; it is now clear in profile and can be measured (Fig. 2C, c). By day 121, most embryos had distinct lobes that would become the antennules, antennae and mandibles. Optic lobes are diffuse and indistinct, and the abdomen is a diffuse round lobe at the base of the "V". By day 128, most embryos have distinct mandibles, and by day 143, the optic lobes are clearly defined. By the end of this stage, the antennules and antennae are elongated, and the abdomen is distinct. This stage corresponds to V-6, and is similar to stage 31 (metanauplius) of Nakanishi (1987) (abbreviated as N-31), or to stage 6 (pre-nauplius) of Moriyasu and Lanteigne (1998) (abbreviated as ML-6).

#### *Stage 4 (Prenauplius)*

Defined by a single observation on day 157 (Fig. 2D, d). The optic lobes are large and rounded. Rudiments of the antennules and biramous antennae are clearly defined, the latter with a medial epipodite. The mandible is forming medial to the antennae. The abdomen is folded over the embryo for about half of its length. Size and shape of the embryo is identical to that of Stage 1, equivalent to stage V-7 and similar to N-33 or ML-8.

#### *Stage 5 (Metanauplius)*

Observed on day 171 only. Optic lobes extend lateral to the rest of the embryo. The tail is about two-thirds the length of the embryo. The telson is forked, but setae are not apparent. Maxilliped rudiments are barely visible lateral to the tail. Embryo area and diameter surpass the starting values. Included in V-7, similar to N-38 or ML-9.

#### *Stage 6 (Eye Formation)*

The eyes are large, lightly pigmented, and extend almost to the edges of the egg (Fig. 2E, e). The telson has 6 or 7 spines (or setae) and reaches the anterior margin of the optic lobes. Lateral appendages have setae. Up to four chromatophores can be seen. Similar to N-42 (day 201) or ML-10.

#### *Stage 7 (Chromatophore Formation)*

This is a period of rapid eye growth and formation. The eyes changed from strongly pigmented crescents (Fig. 2F, f), to being oval-shaped (Fig. 2G, g). Six to eight chromatophores are visible on each side. Maxillipeds are elongated with rudimentary setae. In side view, the embryo takes up one-third of the egg. The telson extends past the optic lobes. Similar to N-44, this stage and the next are included in stage V-8.

#### *Stage 8 (Diapause)*

Yolk is divided down dorsal midline into left and right halves, as well as distinct anterior (pinkish) and posterior (orange) lobes (Fig. 2H, h). The embryo is crescent-shaped and wraps three quarters of the way around the yolk, covering the entire surface in ventral view. Embryo area and diameter reach a "plateau," and do not increase further until after day 329. Growth rate of eye length slows down, and eye width levels off. Heartbeat becomes distinct.

#### *Stage 9 (Eye Enlargement)*

Embryo takes up >50% of egg in side view (Fig. 2I, i). Posterior lobe of yolk is visibly reduced, relative to anterior lobe. Yolk lobes are clearly separated in dorsal view (Fig. 2J, j). This stage is equivalent to V-9.

#### *Stage 10 (Rapid Growth Phase)*

Area, length and width of embryo increase rapidly. Dorsal edge of yolk is separated from the perimeter of the embryo case. All measured dimensions start to increase. Equal to V-10.

#### *Stage 11 (Yolk Depletion)*

Area of yolk decreases rapidly, as other dimensions increase during this period of rapid growth. Maxillipeds are well defined and pigmented (Fig. 2K, k). Equal to V-11.

#### *Stage 12 (Hatching)*

Hatching starts. Embryo length and width, and eye length and width reach maximum values. Ommatidia develop a greenish-yellow fringe, producing a "halo" effect around eye. PAY reaches lowest value. Anterior and posterior regions of yolk become distinct, and the latter is reduced to individual lipid globules. The presence of hemocyanin gives the embryo a bluish tinge. Day 395 (Fig. 2L, l) is midpoint of hatching period. Equal to V-12.

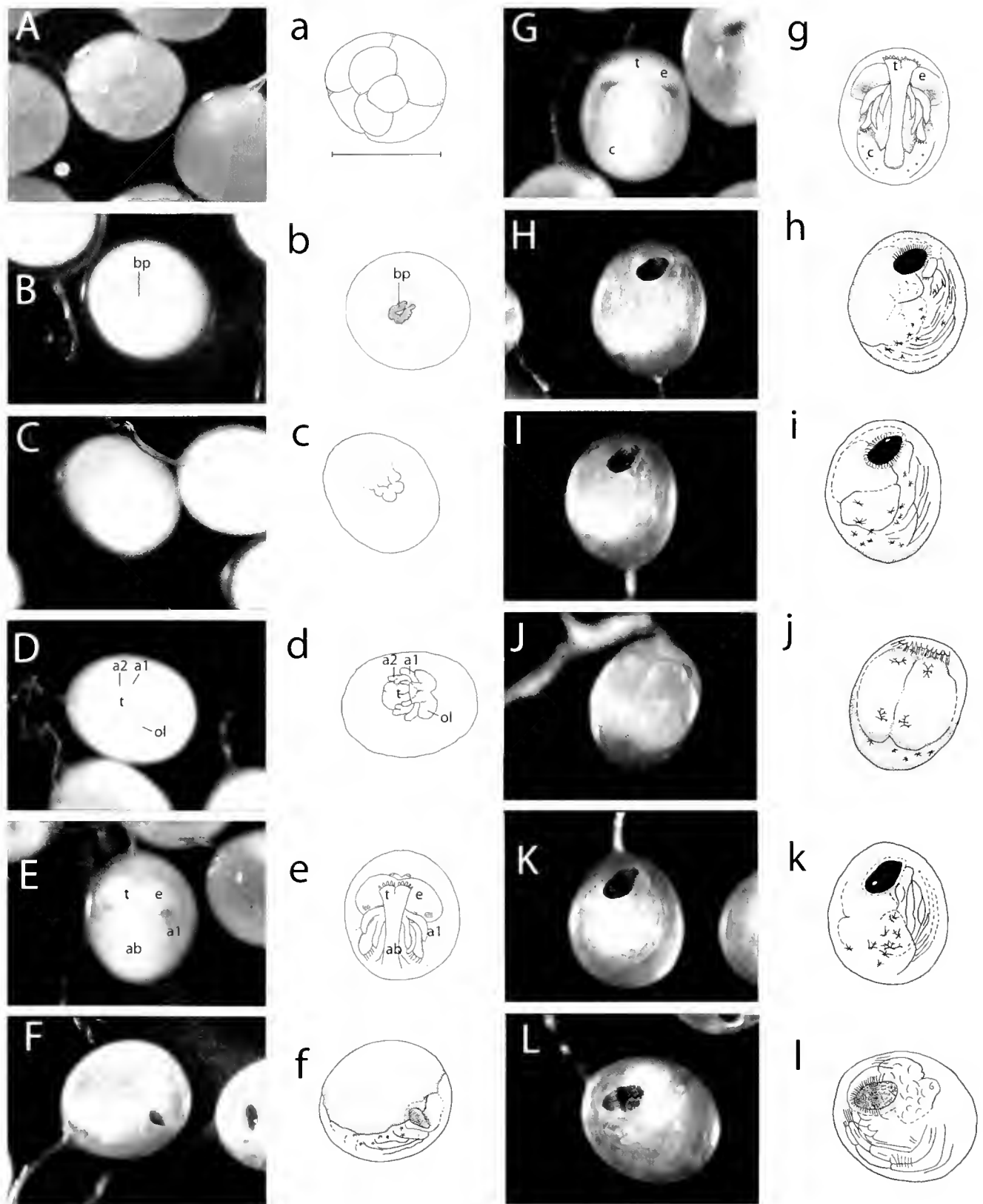


Figure 2. Blue king crab embryo development based on morphometric staging: A, (a) Stage 1, day 7, 16 cells (scale bar = 1.0 mm); B, (b) Stage 2, day 28, blastodisc, stained; C, (c) Stage 3, day 114, "V"-embryo, stained; D, (d) Stage 4, day 157, stained; E, (e) Stage 6, day 192, stained (note setae on maxillipeds and telson); F, (f) Stage 7, day 206; G, (g) Stage 7, day 206, stained; H, (h) Stage 8, day 268; I, (i) Stage 9, day 305; J, (j) Stage 9, day 305, dorsal view showing divided yolk; K, (k) Stage 11, day 367; L, (l) Stage 12, day 395, batching. Abbreviations: a1, antennule; a2, antennae; ab, abdomen; bp, blastopore; c, carapace; e, eye; ol, optic lobe; t, telson.

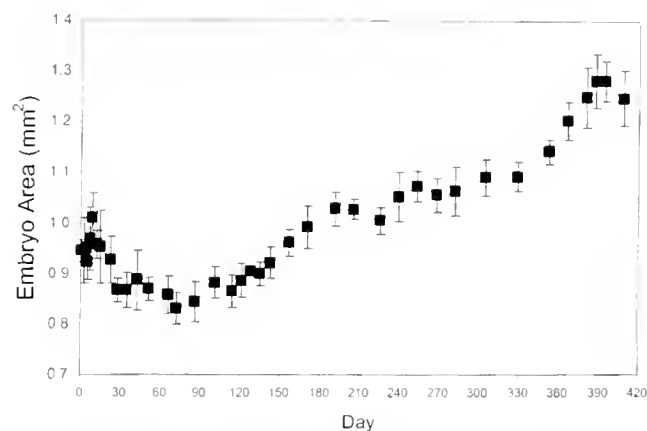


Figure 3. Mean  $\pm 1$  SD of area for blue king crab (*Paralithodes platypus*) embryos, from fertilization to hatching.

#### Morphometric Changes

When first extruded, the mean area of eggs was  $0.95 \pm 0.02$  mm<sup>2</sup> (Fig. 3), and length and width were  $1.17 \pm 0.05$  mm and  $1.03 \pm 0.03$  mm, respectively (Fig. 4). Mean area gradually declined to a minimum of  $0.83 \pm 0.03$  mm<sup>2</sup> on day 72 then began increasing steadily. Length and width followed similar trends. Embryo area, length and width reached a plateau between days 240 and 329 and then increased rapidly from day 353 to day 381, when hatching started. Maximum values were reached for embryo length ( $1.37 \pm 0.02$  mm) on day 381, for embryo area ( $1.28 \pm 0.05$  mm<sup>2</sup>) on day 388, and for embryo width ( $1.19 \pm 0.03$  mm) on day 395. In profile view, yolk occupied 100% of the area of the egg until day 121, when the embryo first became apparent, and PAY was 97.8% (Fig. 5). Yolk area declined as the embryo grew, with a steep decline between days 157 and 206. By day 305, PAY was <50% of total profile area. From day 353 to day 381, during the last month before hatching started, PAY declined most rapidly, from 37% to 12.5%. PAY leveled off during hatching, but reached its lowest value of 12% on day 395. Eye pigment was first observed on day 192, when length and width of the pigmented area were  $0.155 \pm 0.017$  mm and  $0.077 \pm 0.011$  mm, respectively (Fig. 6). Eye length and width both increased steadily thereafter, with the rate of increase leveling off after day 305. Eye length and width increased rapidly after day

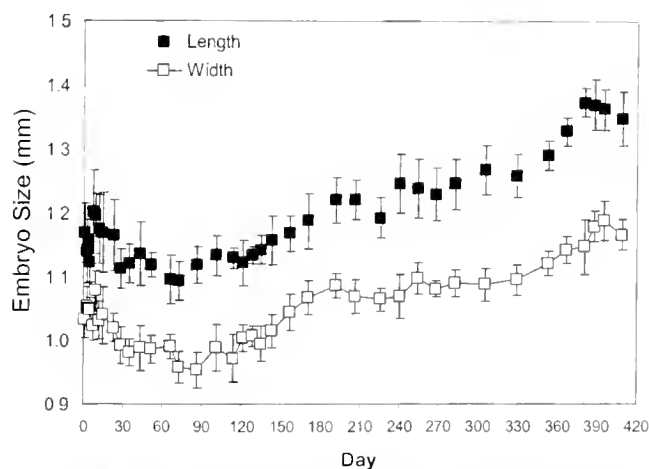


Figure 4. Mean  $\pm 1$  SD of minimum (width) and maximum (length) diameters for blue king crab (*Paralithodes platypus*) embryos.

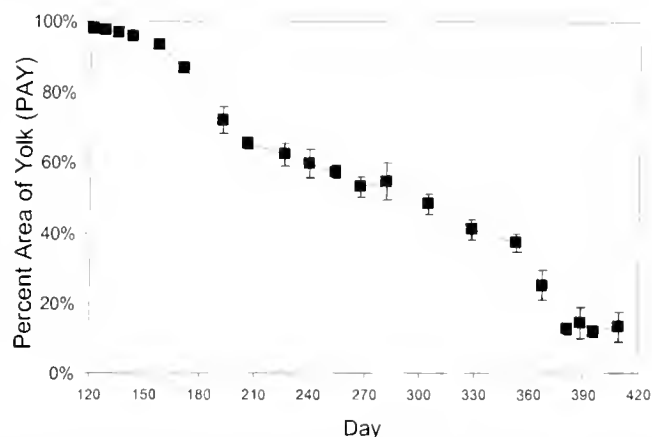


Figure 5. Mean  $\pm 1$  SD of percent yolk visible in side view of blue king crab (*Paralithodes platypus*) embryos.

353, reaching maximal values on days 395 ( $0.431 \pm 0.012$  mm) and 388 ( $0.265 \pm 0.016$  mm) for length and width, respectively.

#### Stage Classification

A total of 39 samples (dates of observation) were used for the analysis. Table 1 defines the stages, starting and endpoint dates (as day number), duration of each stage in days, and percent of total development, as defined by both the traditional (visual) method and the morphometric (clustering) method. The best results were obtained after eliminating the circularity and ellipticity metrics, which showed no linear trend over time. Similar results were obtained regardless of whether calculations were made using PAY as raw data, or after angular or log transformation. Clusters were selected in more-or-less chronological order, even though day number was not used as a variable. Twelve stages were defined by both methods, those stages found to be similar by both methods appear on the same line. In the cluster analysis, a sharp change occurred in both statistical guidelines (the pseudo-*F* and pseudo-*r*<sup>2</sup> values) after 11 clusters. In the dendrogram (Fig. 7), a horizontal line drawn at an average Euclidean distance of about 0.15 (on the vertical axis) would cut across 11 vertical branches, each of which defines a cluster. Two clusters (days 9 and 157) were misplaced, so the former was combined with Cluster 1 and the latter removed

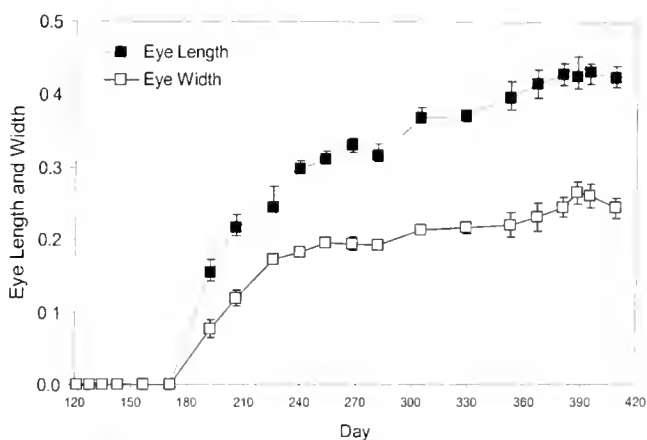


Figure 6. Mean  $\pm 1$  SD of length and width for the pigmented eye of blue king crab (*Paralithodes platypus*) embryos.

TABLE 1.

Developmental stages of blue king crab. Stages were defined either by the traditional visual method, or by analysis of morphometrics. Begin and End are midpoint days between observations and are numbered from fertilization; Days is duration of stage; Percent is duration relative to complete development. Similar stages occur on same lines. Some stages were represented by a single sampling date.

Stages Assigned by Morphometrics		Values at Beginning of Stage								Stages Assigned by Visual Method				
		Begin	End	Days	Percent	Egg Area	Egg Length	Eye Length	Pct Yolk	Stage	Begin	End	Days	Percent
1	Cleavage	0	25	25	6.1%	0.945	1.170			1	0	4	4	1.0%
2	Blastula-Gastrula	25	117	92	22.4%	0.867	1.114			2	4	26	22	5.4%
										3	26	39	13	3.2%
										4	39	108	69	16.8%
										5	108	125	17	4.1%
3	V-embryo	117	150	33	8.0%	0.886	1.122		0.980	6	125	139	14	3.4%
4	Pre-nauplius	150	164	14	3.4%	0.961	1.168		0.935	7	139	182	43	10.5%
5	Meta-nauplius	164	182	18	4.4%	0.992	1.188	0.000	0.870					
6	Eye formation	182	199	17	4.1%	1.028	1.220	0.155	0.721					
7	Chromatophore formation	199	233	34	8.3%	1.028	1.221	0.216	0.655	8	182	233	51	14.4%
8	Diapause	233	294	61	14.9%	1.051	1.247	0.298	0.597					
9	Eye enlargement	294	340	46	11.2%	1.091	1.267	0.368	0.484	9	233	294	61	14.9%
10	Rapid growth	340	360	20	4.9%	1.142	1.291	0.397	0.374	10	294	341	47	11.4%
11	Yolk depletion	360	374	14	3.4%	1.203	1.328	0.415	0.252	11	341	374	33	8.0%
12	Hatching	374	410	36	8.8%	1.249	1.374	0.428	0.126	12	374	410	36	8.8%
					100.0%									100.0%

from it. One additional cluster was further split posthoc into Clusters 2 and 3, leaving 12 useful clusters as stages.

Embryos from three test crabs were classified into three different stages relative to those of Crab #1. Embryos from Crab #2, incubated at 2°C (167 d old) were developmentally delayed because of colder temperatures and were classified as stage 3 (between days 128 and 135 for Crab #1). Embryos from Crab #3, raised at 4°C (192 d old), were classified as a distinct cluster between stage 5 (171 d) and stage 6 (192 d). Embryos from Crab #4 (6°C, 223 d) were more advanced because of warmer temperatures and were classified as a distinct cluster between stages 8 (282 d) and 9 (305 d).

### Hatching

Hatching of larvae was first observed on February 2, 2005, but the female crab was not placed into the isolation tub until February 4, because of space limitations, and larvae were first collected the next day. Therefore, it is likely that several days of larval hatching were missed. On the first night of larval capture 35 mL of larvae were collected (Fig. 8), a much larger volume than on subsequent days. Whereas unusual, such spikes occasionally occur when hatching is delayed because of disturbance or disruption of light cycles. Measurable numbers of larvae were collected for 31 d, and the mean date of hatching was February 12 (day 390). If the 2 d prior to the beginning of larval collections (after female isolation) are included, hatching lasted for a total of at least 33 d.

### DISCUSSION

During early stages, BKC embryos developed at almost the same rate as described for red king crab by Nakanishi (1987), who reported that cleavage was first seen on day 4, a distinct 2-cell stage was not observed, the 4-cell stage appeared on day 5, 8-cells on day 8, and cell numbers doubled daily thereafter. Development of BKC embryos slowed between days 240 and 330 (mid September to mid December) and then increased rapidly until hatching.

The total length of development was longer (390 d to the mean hatch date) than that for 12 primiparous red king crabs (365 d) or for 19 multiparous red king crab (326 d) that were held at an average temperature of 6°C (Stevens & Swiney, in press). Length of hatching, although possibly underestimated at 33 d, was slightly longer than the mean of 28 days determined for 23 BKC in 2004 (Stevens, in press), yet similar to that for red king crabs, which averaged 31 d regardless of parity (Stevens & Swiney, in press). Snow crabs in the Gulf of St. Lawrence also have a 2-y spawning cycle like BKC, but embryos require 2 y to develop (Moriyasu & Lanteigne 1998), versus 13 mo for BKC. Moriyasu and Lanteigne (1998) described 14 developmental stages that roughly correspond to the 12 identified for BKC, plus two earlier stages prior to, and during, funiculus formation; analysis of morphometry did not distinguish such stages in BKC embryos. Nakanishi (1987) examined red king crab eggs at more-or-less weekly intervals, and subsequently described 53 stages.

Crustacean growth and development rates increase with temperature, as has been shown for larval stages of both red (Nakanishi 1981) and southern king crab (Anger et al. 2004), and snow crab (Kogane et al. 2005) and embryos of northern shrimp *Pandalus borealis* (Brillon et al. 2005). Crustaceans from warmer water environments typically have shorter embryonic development on the order of days to weeks. With short developmental periods, observations made at daily intervals are often different enough to be characterized as individual stages. For example, embryos of the fiddler crab *Uca lactea* require an average of 15.4 d to develop, reach the 32-cell stage within 24 h and can be categorized into 15 distinct stages (Yamaguchi 2001). Embryonic development of the redear crayfish, *Cherax quadricarinatus*, requires 42 d at 26.0°C, and was categorized into 10 prehatching and 3 posthatching stages (Garcia-Guerrero et al. 2003). Unlike crabs in the families Lithodidae and Paguridae, *Aegla platensis*, a riverine anomuran in Brazil, develops through the zoea and decapodid stages (equivalent to the megalops or glaucothoe) inside the egg and hatches after 35 d (Lizardo-Daudt and Bond-Buckup 2003).

Because of the great disparity in development time, there is no

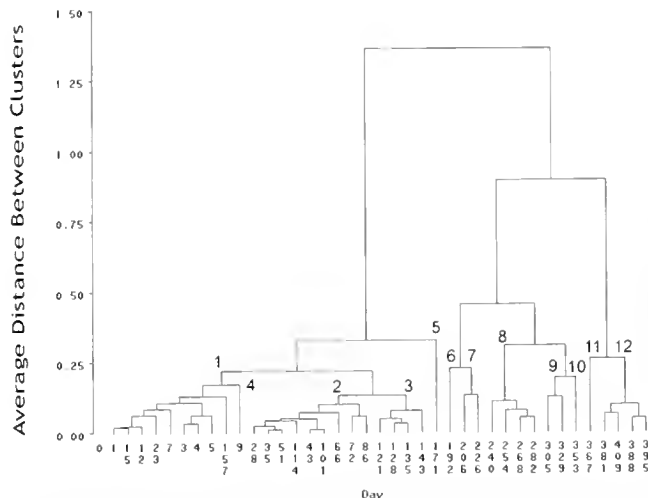


Figure 7. Dendrogram of blue king crab (*Paralithodes platypus*) embryo stages as defined by cluster analysis. 12 clusters are identified, one of which (#4, day 157) was misclassified.

standardized scheme for characterizing developmental stages of crabs or any other decapod crustacean. The number of described stages ranges from 10–15, depending on the length of development and the utility of each stage in describing changes observable by eye. The use of embryo morphometrics is a more quantitative method, and may be a useful approach for comparing development between different populations, environmental conditions or species. Lavarias et al. (2002) used embryo morphometry to describe development in the freshwater prawn *Macrobrachium borellii* and found that predetermined stages could be identified using four metrics, but they did not use a multivariate approach to classify stages based on their similarity. In order for this approach to be useful, however, it requires a larger number of samples than the number of expected stages. Therefore, for species with short development periods, multiple samples per day would be required. However, stages defined by multivariate analysis, whereas relatively easy to construct using morphometric measurements, are not a substitute for examining the embryo by eye to determine the relative development of various appendages, chromatophores and other organs, and some samples (e.g., day 157) could not be accurately classified without visual observations. Examination of embryos from the three extra crabs, whereas not definitive because

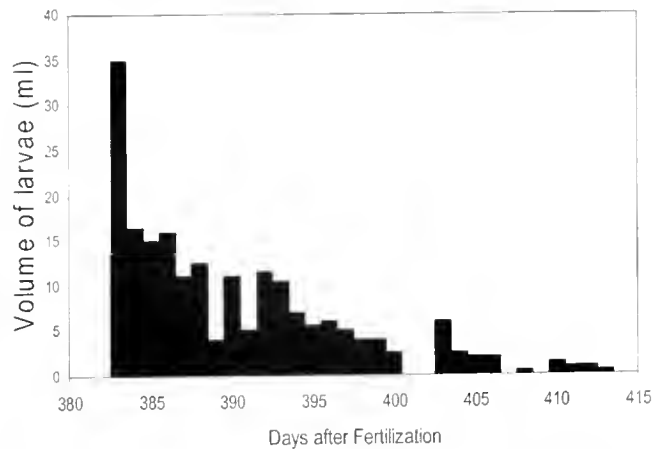


Figure 8. Total volume of larvae hatched each day by the single blue king crab (*Paralithodes platypus*) used in this study.

of the different holding temperatures, does indicate that unknown embryo samples can be classified on a relative scale of development. A more definitive system could be developed by employing classification and regression tree (CART) analysis to define discrete developmental stages based on specific criteria.

The techniques of morphometric analysis used in this study are partly adapted from those used in the medical and veterinary sciences for classifying the “quality” of sperm cells (Verstegen et al. 2002). Automated techniques such as computer-assisted sperm analysis (CASA) (Verstegen et al. 2002) and automated sperm morphometry analysis (ASMA) (Hidalgo et al. 2005) could provide new insight into the study of crustacean embryo development, revealing differences too subtle to be detected by the naked eye that may result from environmental change, anthropogenic disturbance or pollution. Morphometric classification of gametes and embryos may also be useful for identifying and selecting high-quality brood stock for the aquaculture industry or for preservation of endangered species.

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## TIMING AND SEASONALITY OF THE TERMINAL MOLT AND MATING MIGRATION IN THE SPIDER CRAB, *MAJA BRACHYDACTYLA*: EVIDENCE OF ALTERNATIVE MATING STRATEGIES

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**ABSTRACT** Timing and synchronization at individual and population levels of the processes related to the terminal molt, gonad maturation, accumulation of energy reserves and migration in the spider crab *Maja brachydactyla* are analyzed. Also, the intra and intersexual variability is established. Two hypotheses are tested to explain the temporal and population variability: (1) physiological hypothesis: males and females begin migration when they reach the appropriate physiological stage (the optimum level of energy reserves) and (2) mating opportunities hypothesis: the timing of the migration maximize mating opportunities and mate quality. Our results show that males carry out the terminal molt before females, the former having a peak in July and the latter in August. The onset of gonad maturity in females occurs between two and three months after they have reached morphometric maturity (starting in October), coinciding with the period prior to and during the mating migration to deep waters. In an analysis of the spermathecae of primiparous females, it was found that practically no mating activity occurred in shallow waters, whereas the first copulations took place in the migration corridor. However, probably most of the mating activity occurs in deep mating grounds. Males reached gonad maturity prior to morphometric maturity. No differences were observed in the physiological status (muscle, gonad and hepatopancreas relative mass) between migrating and nonmigrating crabs, which is a clear indication that the physiological hypothesis does not hold true for this species. A higher percentage of postmolt crabs were caught in the migration corridor than in shallow waters. The physiological condition improved over time in males and females in the specimens caught in the shallow area as well as in the migration corridor. Therefore, the crabs that start migrating first did so in poorer physiological condition. Moreover, the early migrators had a significantly lower mean size than the late migrators. Our results suggest that variability within populations and between sexes are related to the different reproductive strategies and not to a physiological limitation. Thus, the poor-quality males (with a reduced competitive ability) would migrate at the beginning of the season to maximize the mating opportunities that would not be feasible if they had to compete with the late migrators, which are larger in size and in better physiological condition. Sperm competition is not clear in *M. brachydactyla*, but all evidences point at last male preference for fertilization of eggs.

**KEY WORDS:** decapod, *Maja brachydactyla*, mating, mating strategies, migration, reproductive migration

### INTRODUCTION

Juveniles of *Maja brachydactyla* (Bals 1922, see Neumann 1998 for taxonomic status, corresponding to the North Atlantic species previously known as *M. squinado*) live primarily in shallow waters (González-Gurriarán & Freire 1994, Hines et al. 1995, Le Foll 1993, Meyer 1993, Sampedro 2001, Freire et al. 2002). During the summer of their second or third year of life (depending on the recruitment season) these crabs carry out the terminal molt, associated with the onset of sexual maturity [Corgos 2004, Corgos & Freire (in press), Corgos et al. (Submitted), Freire et al. 2002, González-Gurriarán et al. 1995, Le Foll 1993, Meyer 1993, Sampedro et al. 1999]. Between late summer and early autumn adult specimens carry out a reproductive migration to deep waters as has been observed in different areas on the European coast (Camus 1983, Edwards 1980, Freire & González-Gurriarán 1998, Freire et al. 1999, González-Gurriarán & Freire 1994, Kergariou 1976, Kergariou 1984, Latrouite & Le Foll 1989, Le Foll 1993, Meyer 1993, Stevcic 1973). Although information is scarce on the mating behavior of the spider crab in the field, based on available evidence mating is known to take place mainly in deep waters, where aggregations could be formed (González-Gurriarán et al. 2002). The terminal molt, gonad maturity, migration and mating are factors that are closely interrelated and play an important role in the life history of the spider crab, because they will determine drastic changes in the spatial structure and demography of the populations and are fundamental in terms of reproductive success.

It has been widely documented that migrating animals store energy reserves before they begin to migrate to ensure the success of the migration (Dingle 1996). In the spider crab, migration entails a drastic change in activity (rapid movements of over 1–10 km · day<sup>-1</sup>, after a juvenile phase with only small-scale movements (10s or 100s m · day<sup>-1</sup>, Bernárdez et al. 2005, Freire & González-Gurriarán, 1998; González-Gurriarán & Freire, 1994; González-Gurriarán et al. 2002; Hines et al. 1995). This migration, however, takes place after the terminal molt—a physiologically and energetically critical process. Therefore, during a short period directly after the molt and prior to migration, the processes related to the recovery after ecdysis and the storage of the energy needed for migration must be carried out. Differences between males and females in the timing of migrations in other geographic areas have been reported; particularly the fact that males start to migrate before females (Stevic 1973). This differential behavior may be related to the reproductive strategies of the two sexes, as has been observed in other vertebrate species and other arthropods (Morbey & Ydenberg 2001).

Spider crab males compete for females, which gives rise to agonistic interactions (information based on direct observations by fishermen of occasional matings in shallow waters), and females may mate with several males (González-Gurriarán et al. 1998). Information based on observations of the mating activity of crabs held in captivity and at sea would indicate that males and females mate in hard carapace condition (no mating has ever been observed immediately after the molt). It is possible for ovigerous females to mate, and courtship prior to copulation does not take place, nor does pre or postcopulatory mate guarding (González-Gurriarán et

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al. 1998). This mating behavior differs from the characteristics defined for other majids (Claxton et al. 1994, Jones & Hartnoll 1997, Sainte-Marie et al. 1999). The fact that females have a hard carapace during mating determines the characteristics of the mating system. On the one hand, it means that the existence of mate guarding aimed at protecting females with a soft carapace from predators is not necessary for these crabs (e.g., Hartnoll 1969, Jivoff 1997a, Wilber 1989) and/or ensuring the paternity of the brood (Diesel 1991, Jivoff 1997a), although postcopulatory mate guarding has been observed in hard-shell females of *Chionoecetes opilio* (Claxton et al. 1994). On the other hand, in the case of the spider crab, all the adult females are receptive, which means that the operative sex-ratio, defined as the number of sexually active males versus the number of fertilizable females (Emlen & Oring 1977), is equal to the effective sex-ratio.

In other majids, sperm competition tactics that favors last male's paternity has been observed (Diesel 1990, Rondeau & Sainte-Marie 2001, Sainte-Marie et al. 2000), but this is not clear in *M. brachydactyla* (González-Gurriarán et al. 1998; Freire, unpublished data).

The timing and synchronization of the biological processes associated with reproductive migrations, at the individual and population levels, and the sexual differences observed in the spider crab may be explained by 2 sets of alternative hypotheses:

**Physiological hypothesis:** males and females start migrating when they have reached the appropriate physiological status (an optimum level of energy reserves). Therefore the variability within populations and sexes would be related to the physiological stage, depending on the timing of the terminal molt and the energy recovery rate. Both processes may be variable between sexes, with the energy needed for reproduction, and among body sizes.

The physiological hypothesis could be tested using the following predictions: (1) The physiological condition of the postpubertal adults caught in the migration corridor would be better than that of crabs still remaining in shallow waters. This pattern should be observed throughout the entire migration period. The physiological condition during the migration season would improve gradually in crabs from shallow waters, whereas it would remain relatively constant in time in individuals captured in the migration corridor (Fig. 1). (2) Sexual differences in timing would depend on the differential physiological condition of each sex, related to differences in the timing of the terminal molt.

**Mating opportunity hypothesis:** the timing of the migration should maximize mating opportunities and mate quality. Once a minimum physiological condition is reached, males would migrate at the appropriate time to intercept and mate with the greatest possible number of females in the mating habitats (in the spider crab these would include zones where females may be intercepted during migration and/or in deep waters). The variability within populations and sexes would be related to different reproductive strategies and not to a physiological restriction (possibly once a minimum level of energy reserves has been reached). According to this hypothesis, the intrasexual variability would be determined by the interindividual differences in quality.

The mating opportunity hypothesis could be tested using the following predictions: (1) At any given moment, crabs in migration and in shallow waters would not necessarily exhibit any differences in their physiological condition. (2) Poorer quality males (in the case of crustaceans, animals having a smaller body size and/or lower robust physiological condition) would carry out mi-

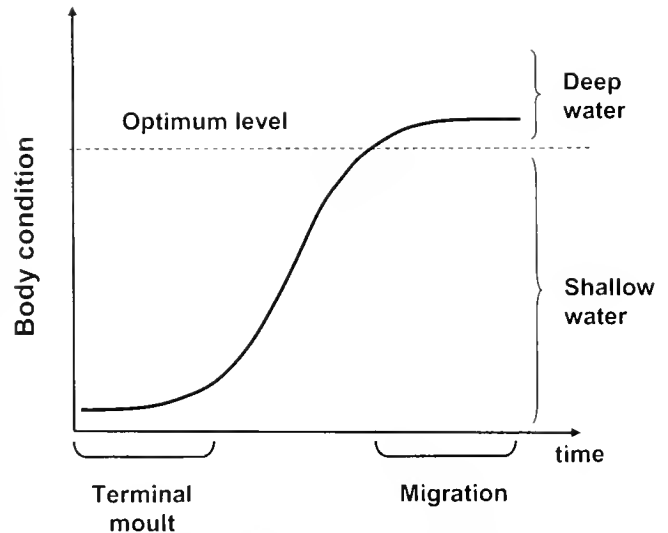


Figure 1. Physiological hypothesis. Temporal evolution of the physiological condition in a cohort of adults after the terminal molt (see Introduction for details).

gration earlier to maximize the possibility of mating encounters before high-quality males arrive to the mating grounds. Therefore, the physiological condition would improve throughout the migration period in both shallow waters and the migration corridor.

From the standpoint of males, mating probability depends on the abundance of females and on the competition among males, which means that the temporal distribution of mating opportunities throughout the season is probably unimodal. At the beginning, mating probability would be low owing to the absence of females in the mating zones. As females start arriving, these odds would likely increase until they reach a maximum value, then the probability would diminish, because the density of males would attain a maximum and the competition among them would cause mating probability to decrease (Fig. 2).

According to the mating opportunity hypothesis, early migrating males would be smaller and have a poorer physiological condition, but they would be the first to arrive at the mating zones. Besides being able to mate with females during migration, they would have a higher probability of mating with the first females to arrive at the mating zones (Fig. 2, A). The density of females would increase over time, but so would the density of males, so the percentage of intercepted females would gradually decline because of the competition (Fig. 2, B), until it reaches a minimum level that would coincide with the maximum density of males (Fig. 2, C).

Late migrating males would arrive at the mating zones later, but they would be larger in size and in stronger physiological condition (high quality males), which means that they would be better competitors than the early migrators. As density of females increases, the mating probabilities of these males would increase (Fig. 2, B) until a maximum value is reached, coinciding with the maximum density of females in the zone (Fig. 2, C).

For females the optimum strategy would be to mate with the males of the best quality available at any given moment. Males with larger ornaments or weapons, greater body size, or higher rates of courtship showed greater survivorship or longevity (Jennions et al. 2001). From this standpoint late migrants should be genetically superior, conveying genetic benefits to females by rising the mean offspring fitness caused by inheritance of "good genes," that may enhance survivorship or longevity of



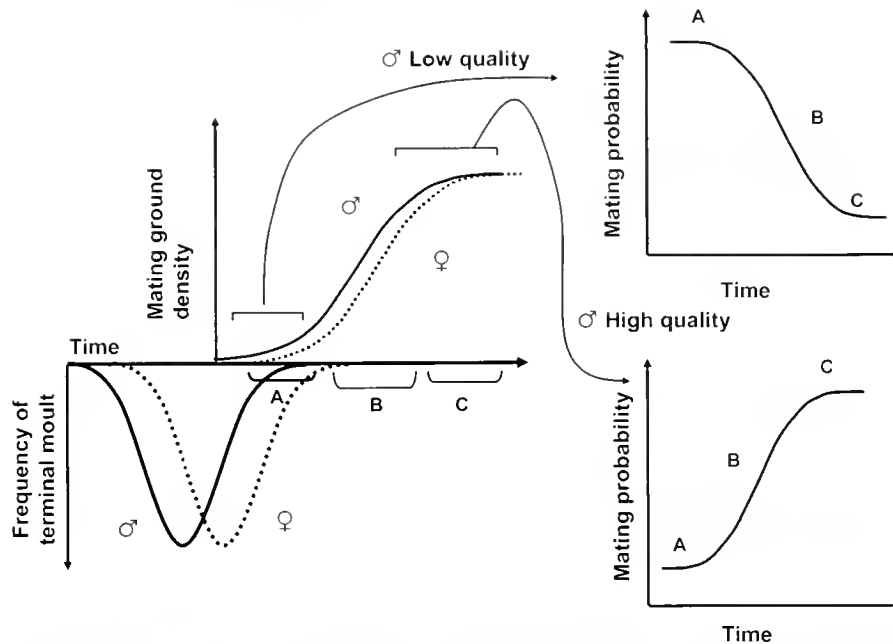


Figure 2. Mating opportunity hypothesis. A, B and C represent 3 periods corresponding to the initial, intermediate and final stages of migration (see Introduction for details).

offspring and increase the sexual attractiveness (Jennions & Petrie 2000).

This study is a detailed analysis of the timing and synchronization at the individual and population levels of processes related to the terminal molt, gonad maturation, accumulation of energy reserves and migration in the spider crab. Intra and intersexual and intrapopulation variability in the earlier mentioned processes are analyzed. Our results will allow comparison, at least in part, the hypotheses proposed earlier related to the causes of the timing and synchronization of the processes linked to migrations.

#### MATERIAL AND METHODS

Monthly samplings were carried out between December 1997 and November 1999. From late summer until autumn, sampling effort was increased to obtain more detailed information on the migration of adults. Sampling was carried out using experimental traps (50-cm height, with upper and lower diameters of 110 cm and 100 cm respectively, and an entry of 22 cm and a 50-mm mesh, Corgos and Freire, submitted a). Soak time was 24 h and traps were baited with fresh horse mackerel (*Scomber scombrus*). The sampling area was the Ría de A Coruña, a small oceanic bay located off the NW coast of Galicia (NW Spain). One shallow water (5–15 m) sampling station was selected in the inner area of the ría (Bastigueiro) and another one in deeper waters (25–30 m) in the central channel of the ría, which constitutes the migration corridor for postpubertal adults. In the inner area of the ría, where there is high abundance of *Maja brachydactyla*, sampling was carried out along a transect on the longitudinal axis of the ría where seven tows were carried out deploying the traps parallel to the coast. Tows were separated approximately 180 m from each other (Fig. 3).

The following data were recorded for each specimen captured: sex, morphometric maturity (Corgos 2004, Corgos & Freire, in

press, Sampedro et al. 1999), stage of the intermolt cycle (estimated by the hardness of the exoskeleton and the presence of a new internal carapace in crabs approaching molt, Sampedro 2001) and the relative age based on the degree of epibiosis and carapace wear (see Fernández et al. 1998), to distinguish the recent postpubertal adults from specimens that had reached maturity in previous years.

To analyze the reproductive stage and physiological condition of males and females, adult females and juvenile and adult males were sampled monthly from July to December 1998 (morphology of the abdomen allow to determine the maturity stage directly in females; Sampedro et al. 1999). The maturity stage of males was determined later based on their morphometry. Samples were obtained from shallow areas and the central channel, although for several reasons in the channel data were obtained only for females in September and November, and for males in November. Males with CL > 60 mm were selected and divided into size classes of 20 mm with a final class of specimens of over 160 mm. A sample made up of a maximum of 10 males belonging to each size class captured in the shallow area was transported to the laboratory. A sample of five males from the three larger size classes was obtained from the captures in the channel, because smaller-sized crabs were not caught in this area. Adult females with a CL > 100 mm were selected and grouped into size classes of 40 mm and a sample of 5 specimens from each size class of crabs caught in the shallow area and the channel was taken to the laboratory.

The crabs were dissected to determine the gonad maturity stage in females (following the classification proposed by González-Gurriarán et al. 1993, González-Gurriarán et al. 1998), gonad dry weight, fullness and the number of sperm masses in the spermathecae in the case of females (following the classification proposed by González-Gurriarán et al. 1993, González-Gurriarán et al. 1998), and the presence of spermatophores and gonad dry weight in males. The gonad, hepatopancreas and the muscle of the second right-hand pereiopod of each specimen were extracted and held for

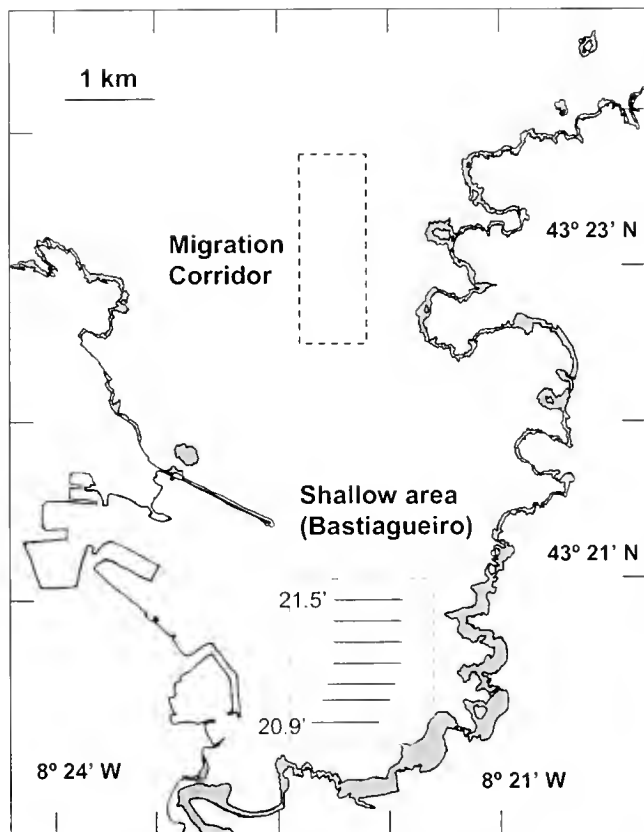


Figure 3. Location and sampling areas in the Ría de A Coruña. The transect comprising 7 trap tows in the inner shallow area is shown. Latitude is given for each tow on the transect (see Figure 6).

48 h at 60°C to obtain the dry weight (Corgos & Freire, in press). Morphometrically juvenile males were omitted from the analysis of the physiological condition.

#### Data Analysis

The energetic status of each individual was estimated by means of condition indices (CI) based on the dry weight of the gonad, muscle and hepatopancreas. The condition indices were estimated as the residuals of an allometric regression ( $\log Y = \log a + b \cdot \log CL$ ) of the dry weight of each type of tissue respect to the carapace length (Fig. 4). The regressions were fitted to the data from the adult specimens caught between June and December 1998. Multiparous adults and juveniles were not included in the analyses.

The differences in the condition indices between migrators and nonmigrators over time, according to the predictions from the hypotheses, were analyzed using the time series corresponding to the condition indices of the crabs captured in shallow waters was fit to a second order polynomial regression:  $CI = a + b \cdot \text{date} + c \cdot \text{date}^2$ . Next, the condition index from specimens captured in the channel (these data were only obtained in some of the months) were compared with the trend of data from Bastiaqueiro. Significant differences between the two areas were accepted when the mean CI of the channel fell outside the 95% confidence band of the regression for the shallow area.

The molt stage was used as an additional body condition indicator, because the crabs in postmolt are in poorer condition than

those in the intermolt or premolt stage. The differences in the molt stage between migrators (early and late) and nonmigrators were determined by means of a log-linear analysis on the frequency of males and females in postmolt in Bastiaqueiro and in the channel in September and October 1998.

An analysis of variance (ANOVA) was performed to test differences on the mean body sizes of the migrators and nonmigrators, using data from males and females caught in Bastiaqueiro and the channel in September and October 1998.

## RESULTS

### Seasonality and Sexual Differences in the Terminal Molt

Adult males in postmolt began to appear in the catches from Bastiaqueiro in April, with their abundance undergoing a rapid increase, reaching high levels in June and a maximum in July. In September, the abundance of postmolt adults dropped sharply and continued to decrease more gradually until the complete disappearance of these crabs in December (Fig. 5). Postmolt females started to be caught in shallow waters in July, attaining maximum abundance in August. They dropped sharply in September and had practically disappeared by October (Fig. 5). The pattern was repeated in 1998 and 1999 and males were found to carry out the terminal molt one month earlier than females. The maximum numbers of postmolt adult males and females were found in July and August respectively. The terminal molt period in males lasted longer than in females (postmolt males were found from April until November in 1998). In 1999, there would seem to be greater synchronization, because adult males started to be caught in June.

### Gonad Development and Mating

Adult females showed a clear seasonal pattern of gonad maturation. After the terminal molt, females had gonads in the early stages of development (between July and October 93% of the females were in stage I) and until November females did not attain more advanced developmental stages (II and III). No females were found with gonads in an advanced stage of development (IV) in either the shallow area or in the channel. In the channel 64% of the females had gonads in stage II and 27% in stage III between November and December. Therefore in females the gonads began to mature between two and three months after the terminal molt, during the period immediately proceeding or during their migration to deep waters.

The analysis of the spermathecae of primiparous females revealed that the number of matings in the shallow zone was low, because 97% of females ( $n = 124$ ) showed empty spermathecae. An increase in mating frequency was observed in the channel, given that over 21% of the females studied ( $n = 23$ ) had mated. These results would indicate that mating may be carried out in the migration corridors, but most likely it is in the deep waters where most of the mating activity takes place (Table 1). All the females analyzed exhibited a single sperm mass. In the shallow area, in contrast, two multiparous females that were captured had 7 sperm masses in each spermatheca, presenting differences in color and volume, which would point to the existence of long intervals between matings (González-Gurriarán et al. 1998).

After examining the gonads of the male crabs, it was evident that, unlike the females, gonad maturation takes place prior to morphometric maturity. Over 60% of the juveniles examined ( $n =$

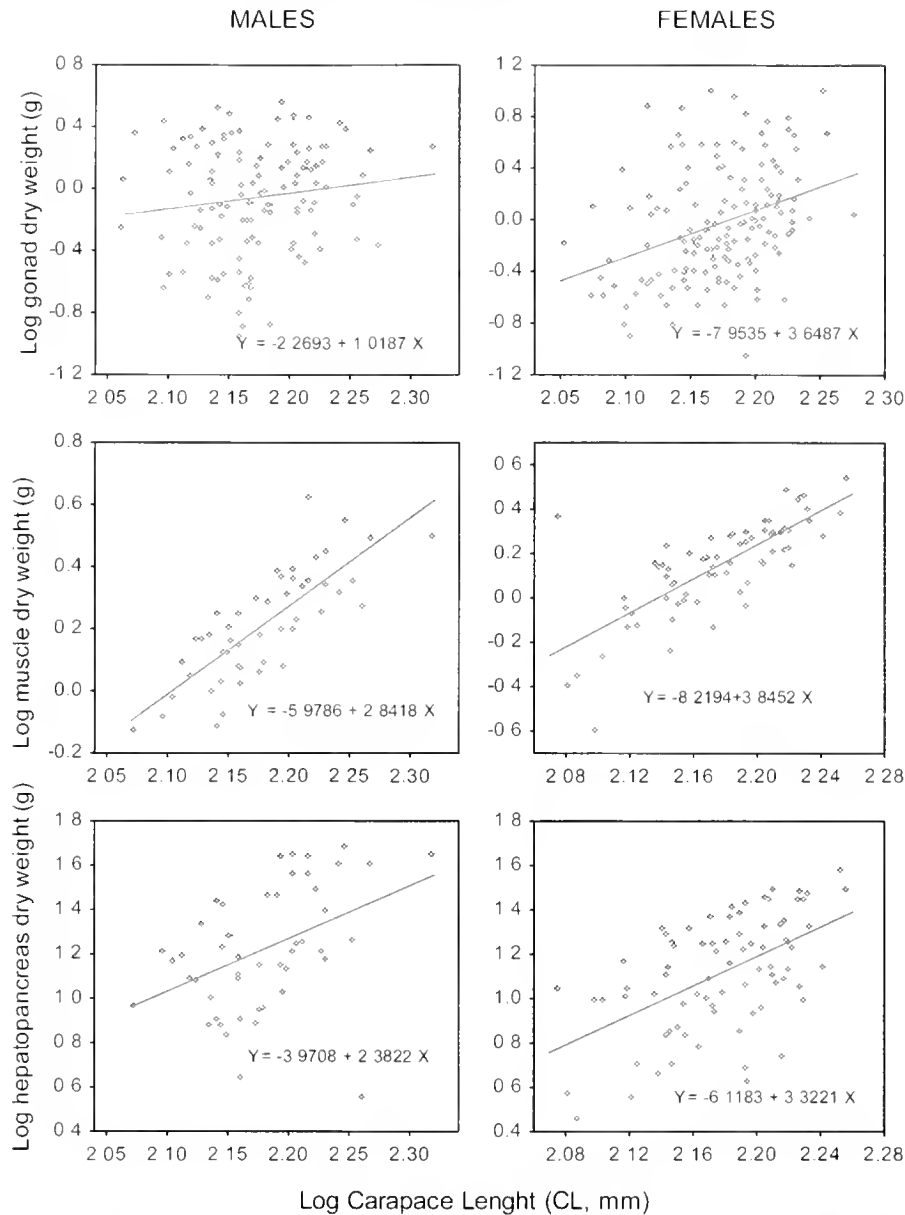


Figure 4. Relation between body size and dry weight of the tissue analyzed (gonad, muscle and hepatopancreas) to estimate the physiological condition of each sex. The allometric regression fitted in each case is shown.

382) had spermatophores, and more than 76% of the juveniles with a CL > 96 mm as well as all of the morphometrically mature crabs had spermatophores. Therefore, males are functionally mature and ready to mate immediately after the terminal molt (see Corgos & Freire, submitted a).

#### *Sexual Differences in the Timing of the Start of Migration*

Based on the sampling carried out in the inner ría, it was possible to carefully monitor the small-scale movements of the adults and the start of their migration (Fig. 6). In July 1998 the mean CPUE of males was much greater than that of females (4.2 and 0.5 crabs · trap<sup>-1</sup> respectively). Males were also found to be sparse around the innermost part of the ría and near the shoreline.

In August the average CPUE of females found in the inner ría increased (3.8 crabs · trap<sup>-1</sup>), and males had moved to the outermost zone near the channel. By September, the large concentration of males in the outer part of the inner area had disappeared, which would imply that they had started migration, whereas the females had moved to the outer zone. In October, the few males left in the area started moving to the outer zone and leaving the area, whereas the females were concentrated in the outer part of the shallow area. In November, the catches yielded few adults, although some females were still found in the outermost area, which means that the females migrated between October and November. By December practically all the adults had left the area.

The catches taken in the channel confirm this seasonal migratory pattern (Fig. 7). They were concentrated in the months of

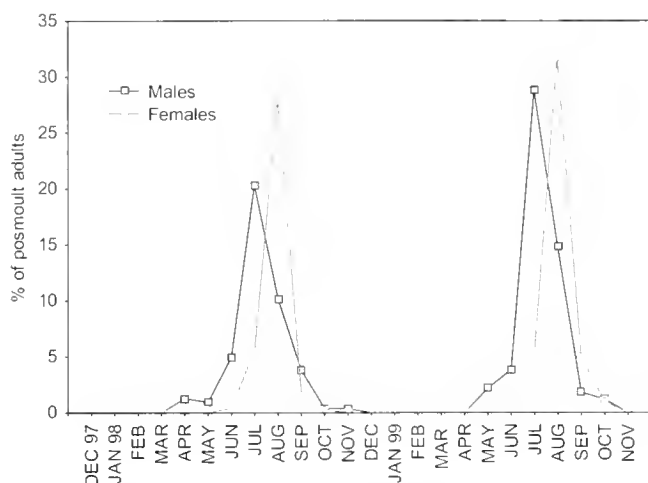


Figure 5. A comparison of the monthly evolution of the proportion of adult males and females in postmolt stage (related to total catches including adults and juveniles) captured in shallow waters.

September and October; however maximum catches of males were obtained in September and those of females in October. Males exhibited stable catches between September 25 and October 23 (Fig. 7). The first females, on the other hand, were caught on

TABLE 1.  
Temporal evolution of spermathecae repletion (percentage of specimens with sperm) of primiparous females in shallow water and the in migration corridor of the Ría de A Coruña.

	Shallow Water		Migration Corridor	
	N	%	N	%
Jul 98	20	10.0		
Aug	19	0.0		
Sep	32	0.0	9	0.0
Oct	24	0.0	1	0.0
Nov	15	0.0	11	45.5
Dec	14	7.1	2	0.0
Total	124	2.4	23	21.7

September 25, from which time catches increased gradually until they reached a clear peak on October 23.

#### Body Condition at the Start of Migration

From July to December, adult males in Bastiagueiro underwent a progressive increase in the relative weight of the muscle, gonad and hepatopancreas (Fig. 8). It was only possible to compare the

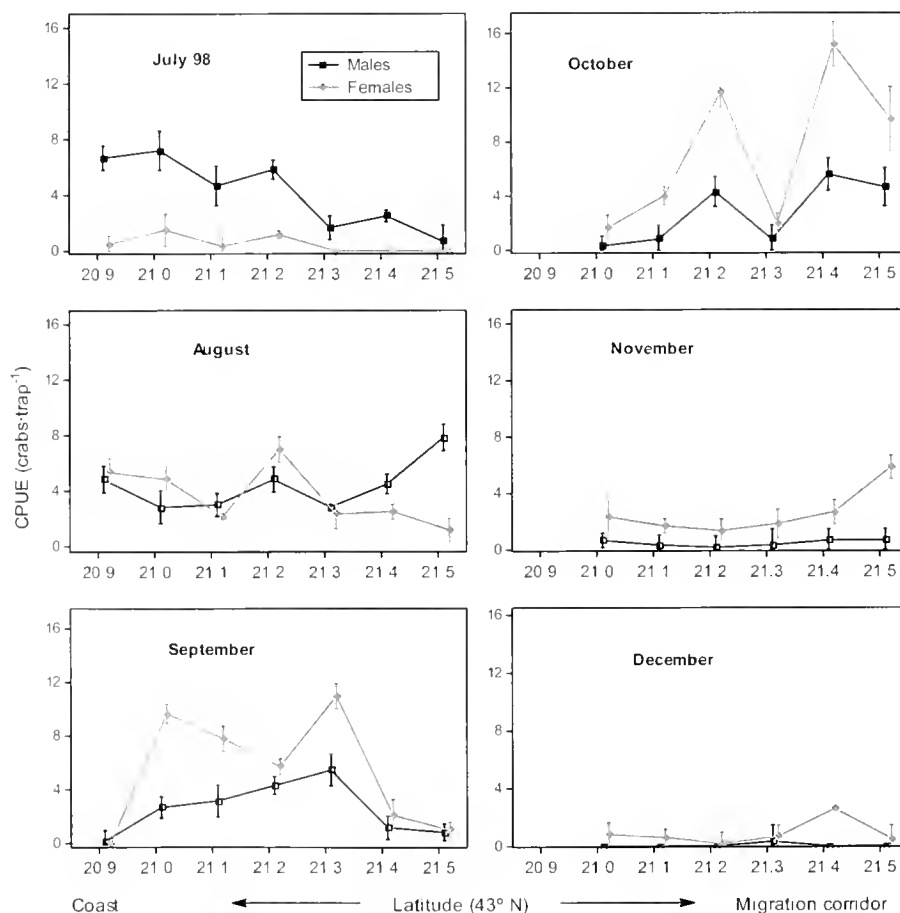


Figure 6. Spatial structure of the adult population in Bastiagueiro in the period prior to and at the start of migration. The catch per unit of effort is given (crabs  $\cdot$  trap $^{-1}$  and a 95% confidence interval of the mean) along the transect sampled (each 10th of a degree is equivalent to 182 m) (see Figure 3).

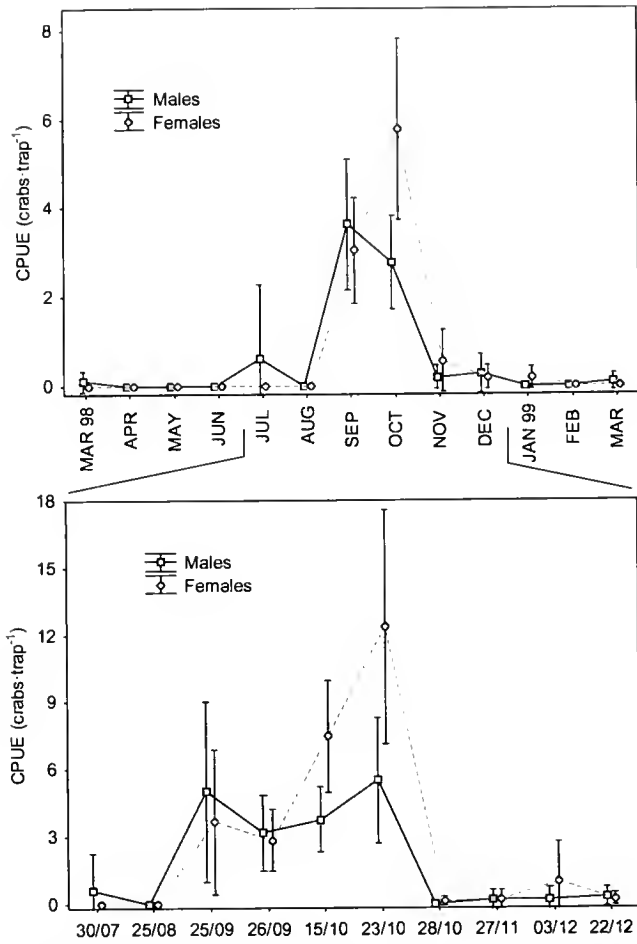


Figure 7. Abundance of adults in the channel (mean crabs · trap<sup>-1</sup> with a 95% confidence interval of the mean) during each month of the sampling (upper), and on each day of sampling during migration (lower) in 1998.

physiological condition in the channel zone (migrators) and shallow area (nonmigrators) in November. During this month migrators presented condition indices similar to those of nonmigrators in all the tissues analyzed.

The condition index in females found in the shallow areas showed a gradual increase in the case of the gonad, but this was not so in the case of the muscle or hepatopancreas (except for very low values in the latter tissue in September). The adult females captured in the channel had slightly higher gonad condition values in November as compared with the shallow zone, whereas no differences were observed in September (Fig. 9). The physiological condition of the muscle was similar in November, which was the only month in which information was available for the channel. The physiological condition of the hepatopancreas, however, was similar in the two areas in September and November.

The percentage of postmolt males and females in each zone was higher in September than October (Table 2), and in both months the percentage was higher in the channel than in Bastiagueiro (log-linear analysis of the effects of sampling station, date and sex on the percentage of specimens in the postmolt stage, showed the significant variables to be sampling station and date,  $X^2 = 5.53$ ,  $P = 0.35$ ). This would imply that the condition of the early migrators is poorer than crabs that migrate later.

#### Body Size at the Start of Migration

The mean size (CL) of males and females was compared between Bastiagueiro and the channel in September and October. Females were significantly larger than males, and in Bastiagueiro as well as in the channel for males and females, the largest sizes were found in October (ANOVA,  $n = 1198$ ; effect of date and sex,  $P < 0.001$ ; effects of sampling station and all interactions,  $P > 0.2$ ). None of the interactions proved to be significant, which was an indication that the smaller-sized specimens of males and females started migrating earlier (Table 3).

#### DISCUSSION

This study provides evidence as to the existence of synchronization in the timing of the terminal molt, gonad development and migration in the spider crab. The inter- and intra-sexual differences related to these phenomena may reflect different types of behavior and reproductive strategies that would be compatible with the mating opportunity hypothesis discussed earlier. However, our results

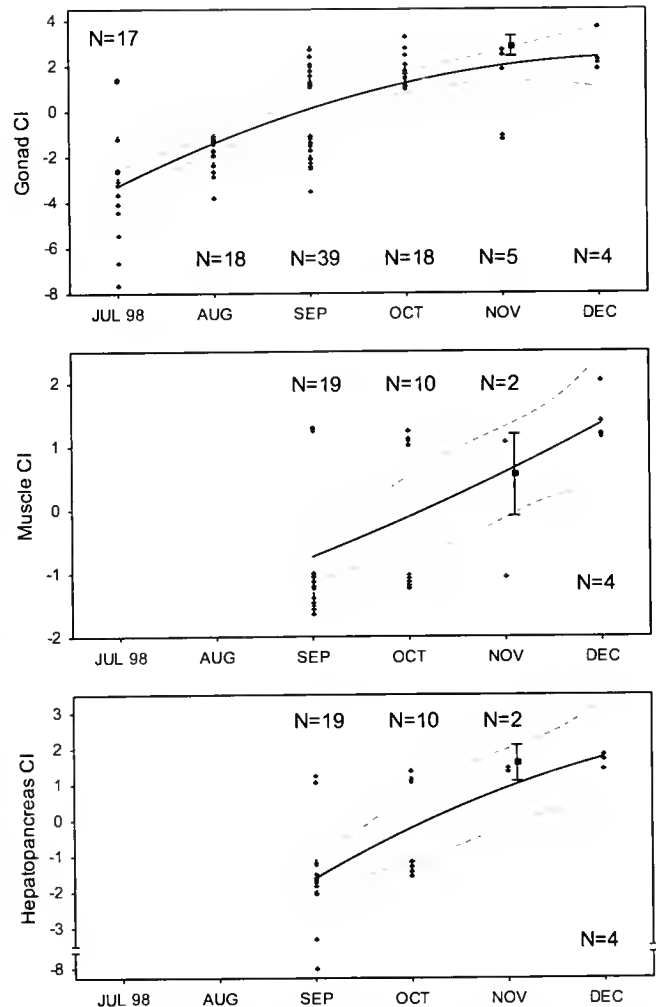


Figure 8. Temporal evolution of the condition index (CI) of each tissue in adult males in Bastiagueiro (individual observations, sample size for each month and polynomial regression with 95% confidence interval are shown) and in the central channel (mean and 95% confidence interval, only in November,  $n = 14$ ).

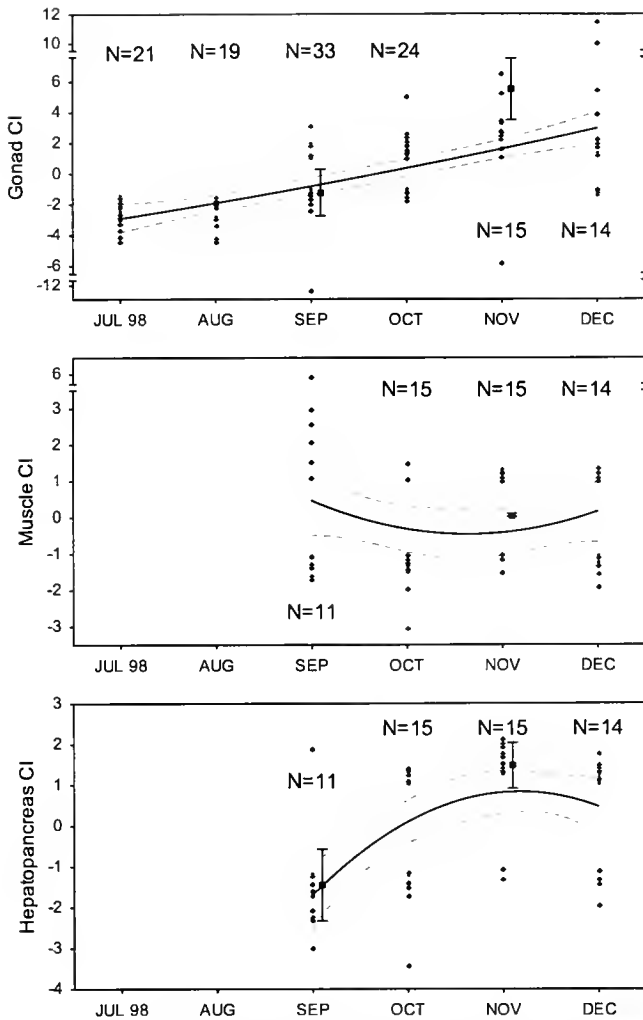


Figure 9. Temporal evolution of the condition index (CI) of each tissue in adult females in Bastiagueiro (individual observations, sample size for each month and polynomial regression with 95% confidence interval are shown) and in the central channel (mean and 95% confidence interval, only in September and November,  $n = 14$ ).

contradict some of the predictions made in the physiological hypothesis.

To mate, an organism must be in the appropriate physiological condition, as well as having a suitable location in space and time (Correa & Thiel 2003). In *M. brachydactyla*, males probably reach maturity and develop gonads before females to be physiologically prepared to mate at the time and place where the encounter with the adult female occurs. Considering the date when migration starts and the fact that in December and January, ovigerous females begin to appear in the catches in the shallow areas (unpublished data), aggregation and mating in deep waters must occur over the course of a short period. Because females have spermathecae, they are able to mate when their gonads are in early stages of development, fertilizing the broods later.

In the Ría de A Coruña, the terminal molt takes place in shallow waters between April and August. Males undergo the terminal molt one month earlier than females. González-Gurriarán et al. (1995) obtained similar results with this species under culture conditions, whereas in more northern latitudes, the cycle is delayed, owing possibly to the differences in the thermal regimen. In the

TABLE 2.

Monthly percentage of adult males and females in postmolt (stage B) captured in Bastiagueiro and in the migration corridor in the migration period. Sample size is indicated in each case.

Date	Sampling Station	Sex	N	% in Postmolt
September 98	Bastiagueiro	Males	129	8.5
		Females	252	2.4
	Migration corridor	Males	87	11.5
		Females	73	9.6
October 1998	Bastiagueiro	Males	94	1.1
		Females	250	0.4
	Migration corridor	Males	97	4.1
		Females	201	4.0

English Channel the crabs undergo the terminal molt in late summer and early fall and adults that have recently reached the post-pubertal stage start to be captured in August-September, presenting maximum catches in early fall (Le Foll 1993, Meyer 1993).

An analysis of the captures in the channel during the migration shows that over the entire period males and females migrated, as would be expected in a species where the females are polyandric (Zonneveld 1992). In view of the captures obtained in the migration corridor, males showed stable catches during the migration period without a specific peak, whereas females, which started migration at the same time as males, exhibited a clear peak on October 23, 1998, after which catches were virtually nonexistent. These results indicate that the migration of males is not as synchronized as that of females, rather males carried out migration gradually over a 30-day period, whereas most females migrated between October 15 and 28, 1998.

Mating takes place generally after migration, in the deep-water wintering habitats, although copulation could occur also during migration (21% of the females caught in the channel had sperm in the spermathecae). These data largely coincide with those reported by González-Gurriarán et al. (1998) in the Ría de Arousa, where the spermathecae of 100% of the primiparous females caught in the shallow zone were found to be empty. On the other hand, catches of multiparous females having several sperm masses, along with direct observations by fishermen, support the hypothesis that mating does occur in shallow waters, but probably re-

TABLE 3.

Mean size (Carapace length, CL, with standard deviation, SD) of the adult males and females captured in Bastiagueiro and in the migration corridor at the start of migration. Sample size is indicated in each case.

Date	Sampling Station	Sex	N	CL	
				Mean	SD
September 98	Bastiagueiro	Males	134	138.9	14.63
		Females	258	148.7	11.25
	Migration corridor	Males	87	139.9	11.19
		Females	72	150.8	11.34
October 1998	Bastiagueiro	Males	95	144.0	14.13
		Females	254	151.4	12.91
	Migration corridor	Males	97	143.0	13.38
		Females	201	152.2	12.30

stricted to multiparous females. In latitudes located farther to the north, matings were observed in shallow waters from May to July on the Irish coast (Brosnan 1981), and starting in June on the French coast (Kergariou 1984). Based on these observations, these authors report that mating generally occurs in summer, although to support this hypothesis, it would be necessary to carry out an analysis of the spermathecae content of females in wintering habitats.

The analysis of catches in the shallow area and the migration corridor and condition indices may be used as indicators of the reproductive strategies of *M. brachydactyla*. The condition indices of the tissues analyzed were similar in the shallow area and the migration corridor (i.e., no differences were observed in the body condition between migrators and nonmigrators), which would clearly imply that the physiological hypothesis is not supported in this case. The physiological condition increased over time in males and females both in crabs caught in Bastiagueiro and the migration corridor. Consequently, crabs that migrate first, do so in poorer physiological condition. Moreover, the early migrators were smaller in size than the late migrators. These evidences support the mating opportunity hypothesis. Poorer quality males (smaller sized and in poorer physiological condition) migrated earlier, which gives them mating opportunities that would not be feasible if they had to compete with the late migrators that are larger in size and in better physiological condition.

In both majids (Conan & Comeau 1986, Elner & Beninger 1995, Ennis et al. 1990, Sainte-Marie et al. 1997, Rondeau & Sainte-Marie 2001, Stevens et al. 1993) as well as other decapods (Correa et al. 2003, Jivoff 1997b, Van Der Meer 1994, Wada et al. 1997) agonistic interactions have been observed between males prior to mating, in which the large-sized individuals exclude the smaller ones. At other times the small males are rejected by the females (Goshima et al. 2000). For this reason the larger-sized males are more likely to mate than the smaller-sized animals. This hypothesis is corroborated by observations in the field, where mating majid males were seen to be generally larger than the females (Brosnan 1981, Conan & Comeau 1986, Ennis et al. 1988, Paul 1992, Powell et al. 1972).

The less competitive (smaller-sized) male decapod crustaceans tend to avoid direct agonistic encounters with larger males (Clark 1997, Ra'anán & Sagi 1985) and they adopt alternative mating strategies, such as intercepting females moving to the mating areas (Van Der Meer 1994) or copulating quickly, whereas two dominant males are competing for a receptive female (Clark 1997, Correa et al. 2003). In this study the less competitive males migrated earlier to the mating zones, probably to be able to copulate before the more competitive males arrived.

In other majids such as *Chionoecetes opilio* (Sainte-Marie et al.

2000) and *Inachus phalangium* (Diesel 1990), the sperm masses are stored dorsoventrally in the spermathecae when they are not too full (i.e., the last sperm mass is stored closer to the oviduct). This stratification favors that the sperm of the last male fertilize the brood, and therefore the paternity is attributed to only one male. In both species males used several strategies to promote their own paternity. Males of *C. opilio* invest more sperm in females that had mated previously than in virgin females to displace and isolate the sperm deposited earlier (Rondeau & Sainte-Marie 2001), whereas males of *I. phalangium* displace previously deposited masses by transferring large amounts of seminal plasma, which hardens forming a gel that completely seals off the previously deposited masses (Diesel 1990). All of these strategies favor the paternity of the last male to mate with a female. In our study, the high-quality males (larger and in better physiological condition) would be the last to mate. In *M. brachydactyla*, however, the arrangement of the sperm masses is different than what was observed in the previously mentioned species. When several masses are present, they are arranged parallel to the main axis of the spermathecae, and if the spermathecae are full, the arrangement is not stratified (González-Gurriarán et al. 1998; Freire, unpublished data). Although all of these authors reported some differences in the size of the masses, they were attributed to different storage time or the possibility of having been used in part to fertilize a brood, and not to the existence of sperm competition. Based on these findings, there is no evidence that the last males to mate (those of better quality) have a greater probability of ensuring their paternity. However, the results of this study seem to suggest that high-quality males will inseminate females after these might have received sperm from low-quality males. This would only be a successful strategy if last males have fertilization advantages in *M. brachydactyla*. Future studies are required to examine whether sperm precedence in this species follows the same pattern as suggested for other majid species (i.e., last male precedence). The present results of diverging tactics of males during mating migration also seem to call for this pattern in *M. brachydactyla*.

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## EFFECT OF DIFFERENT LEVELS OF DIETARY $\alpha$ -TOCOPHEROL ON OVARIAN MATURATION AND REPRODUCTIVE PERFORMANCE OF BROODSTOCK *LITOPENAEUS VANNAMEI* (BOONE)

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**ABSTRACT** Effects of 4 grade levels of dietary  $\alpha$ -tocopherol (37.82, 115.64, 349.42 and 522.56 mg kg<sup>-1</sup> diet) on ovarian maturation and reproductive performance of broodstock shrimp, *Litopenaeus vannamei* were investigated. The  $\alpha$ -tocopherol concentrations in hepatopancreas, ovaries, eggs and muscle of female shrimp increased significantly as dietary  $\alpha$ -tocopherol levels increased. Survival, spermatophore weight, sperm count, gonadosomatic index (GSI), hepatosomatic index (HSI), fecundity and egg diameter were not significantly affected by dietary  $\alpha$ -tocopherol levels. Average daily spawns, hatching rate and fertilization rate significantly increased as dietary  $\alpha$ -tocopherol levels increased. Results of this study confirmed the importance of supplementation of  $\alpha$ -tocopherol to broodstock diets fed to *L. vannamei*, and suggested that at least 350 mg  $\alpha$ -tocopherol kg<sup>-1</sup> of diet was needed to achieve significantly better ovarian maturation and reproductive performance.

**KEY WORDS:**  $\alpha$ -tocopherol, broodstock, *Litopenaeus vannamei*, maturation, reproductive performance

### INTRODUCTION

In penaeid shrimp, broodstock nutrition is a key factor influencing sexual maturation, reproductive performance and offspring quality (Harrison 1990). To develop suitably performing formulated diets for shrimp broodstock, understanding nutrition-reproduction interactions and determining the specific nutrient requirements for successful maturation and spawning are necessary.

Vitamin E (tocopherols) is considered to be an essential dietary nutrient for crustaceans (Conklin 1997). Available data have shown that  $\alpha$ -tocopherol, the most active form of vitamin E, is the predominant form in shrimp/prawn tissue (Cavalli et al. 2001, Wouters et al. 2001), and DL- $\alpha$ -tocopherol acetate, a stable form of  $\alpha$ -tocopherol, is the most commonly used vitamin E supplement in animal feeds (National Research Council 1983). The biological role of  $\alpha$ -tocopherol is widely accepted to be a lipophilic antioxidant, protecting membranes of cells and organelles from oxidation by scavenging organic free radicals (Burton & Trabor 1990), an activity which may be especially important during embryonic and larval development. The importance of vitamin E for fish reproduction has long been recognized (Watanabe & Takashima 1977, Watanabe et al. 1985), but the importance of this vitamin in shrimp reproduction has only been recently demonstrated. Alava et al. (1993) reported that a  $\alpha$ -tocopherol-deficient diet resulted in retarded gonadal maturation of *Marsupenaeus japonicus*. Chamberlain (1988) found a correlation between the percentage of abnormal sperm and dietary vitamin E deficiency in *L. setiferus*. In a trial using *Fenneropenaeus indicus* spawners, Cahu et al. (1995) demonstrated that hatching percentage increased when dietary  $\alpha$ -tocopherol levels increased from 40–350 mg kg<sup>-1</sup>. In wild female broodstock *L. vannamei*, Wouters et al. (2001) detected an increase in  $\alpha$ -tocopherol concentrations in the ovary prior to sexual maturation. All these results suggest that vitamin E plays a critical role in the shrimp maturation process.

Information about the effect of dietary  $\alpha$ -tocopherol on ovarian maturation and reproduction of *L. vannamei* is lacking. In a previous study, we successively substituted a natural diet consisting of

50% bloodworm (*Glycera chirori*) and 50% oyster (*Crassostrea rivularis*) with a formulated diet for *L. vannamei* broodstock (Du et al. 2004a). In the current study, this previously successful formulated diet was used as the basal diet, and ROVIMIX E50 (Roche Sunve Vitamins Ltd, Shanghai, P.R. China) was added as a  $\alpha$ -tocopherol source to investigate the effect of dietary  $\alpha$ -tocopherol on the maturation and reproductive performance of *L. vannamei*. The effect of dietary levels of  $\alpha$ -tocopherol on  $\alpha$ -tocopherol concentrations in ovaries, hepatopancreas, eggs and muscle was also determined.

### MATERIALS AND METHODS

#### Animals and Treatment

Pond-reared *L. vannamei* breeders were obtained from Dongfang Co. (Zhanjiang, P.R. China). They were held in maturation tanks for 3 wk to acclimate to the experimental conditions and fed the basal diet without any supplement of  $\alpha$ -tocopherol acetate (Diet E1). A unisex system as described by Browdy et al. (1996) was used: each of 4 maturation tanks was stocked with 15 females each and each of another 4 maturation tanks with 15 males. They were divided into four groups: E1-E4, each consisting of a male tank and a female tank. After acclimation, female shrimp were unilaterally eyestalk-ablated with a pair of flamed tweezers to enhance the rate of ovarian maturation. To identify each female within the same group, part of the telson was cut with the exception of five females and latter sampled to test gonadosomatic index (GSI) and hepatosomatic index (HSI). The duration of the post-ablation phase of the experiment was 50 days, during which, broodstock shrimp representing treatment E1-E4 were fed diets E1-E4, respectively. All treatments were randomly assigned to the maturation tanks.

The maturation tanks were rectangular-shaped cement tanks (2 × 3 m<sup>2</sup>, 55 cm water depth) in which sand-filtered and UV-treated seawater was exchanged at a rate of 200% daily. The physicochemical parameters of the water were: mean temperature, 28.5 ± 1°C; mean salinity, 30 ± 0.5 mg L<sup>-1</sup>; mean pH, 8.2 ± 0.1; photoperiod, 12 hr light/12 hr dark; and light intensity, 200 lx. Under these conditions, the level of dissolved oxygen remained

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close to saturation;  $6.2 \text{ g m}^{-3}$ , and ammonia, nitrate and nitrite levels did not exceed 0.03, 0.01 and  $0.25 \text{ mg L}^{-1}$ , respectively.

#### Diets and Feeding

Ingredient composition of the basal diet is presented in Table 1. The  $\alpha$ -tocopherol source used was Roche ROVIMIX E50, an  $\alpha$ -tocopheryl acetate containing  $500 \text{ mg } \alpha$ -tocopherol  $\text{g}^{-1}$ . ROVIMIX E50 was added at different levels, compensated by appropriate additions of carboxy methylcellulose as filler (Table 2). Levels of  $\alpha$ -tocopherol added in the dietary treatment were 0, 100, 300 and  $500 \text{ mg/kg}$ . To prepare the diets, all dried ingredients were smashed and sieved, and then thoroughly mixed with 35% (w/w) water. The resulting stiff dough was pelleted with a mincer with a 3-mm die, and dried at room temperature. The dried "spaghetti like" strands were then crumbled to about 5-mm long pieces and kept in sealed plastic bags at  $-20^\circ\text{C}$  until use. Shrimp were fed at a daily rate of 5% of the tank biomass (wet weight), proportionately divided over 4 times daily (8:00, 11:00, 14:00 and 18:00). Uneaten food was collected daily.

#### Broodstock Maturation and Spawning

After unilateral-ablation, females were visually examined for ovarian maturation stages each day at 20:00 according to Wouters et al. (2001). When the majority of the females showed some degree of ovarian maturation, and just before the first mature female with intact telsons was ready to spawn, all females with intact telsons from each treatment were weighed and dissected to determine the gonadosomatic index ( $\text{GSI} = 100 \times \text{gonad weight} / \text{total body weight}$ ) and hepatosomatic index ( $\text{HSI} = 100 \times \text{hepatopancreas weight} / \text{total body weight}$ ).

TABLE 1.

Ingredients and proximate biochemical composition of the basal diet

Ingredients	Weight ( $\text{g kg}^{-1}$ diet)
Fish meal (CIPSSA, Puerto Montt, Peru)	340
Oyster ( <i>Crassostrea rivularis</i> ) meal	200
Bloodworm ( <i>Glycera chironi</i> ) meal	100
Alpha starch§	206.714
Shrimp head meal (50% CP)§	50
Cod liver oil§	40
Soybean lecithin§	15
$\text{Ca}(\text{H}_2\text{PO}_3)_2 \cdot \text{H}_2\text{O}$	10
Cholesterol	5
Sodium alginate	20
Vitamin C (Stay C-35)†	2,286
Vitamin mix*	10
Proximate biochemical composition	(%)
Moisture	$6.01 \pm 0.18$
Ash¶	$15.58 \pm 0.25$
Crude protein¶	$49.66 \pm 0.82$
Total lipid¶	$12.14 \pm 0.49$

§ Alpha starch, shrimp head meal, soybean lecithin and cod liver oil were supplied by Yuehai Feed Co Ltd, China.

† Stay C-35 (Roche Sunve Vitamins Ltd., Shanghai), mainly monophosphate, containing  $350 \text{ mg L-ascorbic acid g}^{-1}$ .

\* Vitamin composition ( $\text{mg/IU kg}^{-1}$  diet): vitamin A palmitate  $100\,000 \text{ IU}$ ; vitamin  $\text{D}_3$   $10\,000 \text{ IU}$ ; biotin  $7 \text{ mg}$ ; ascorbic acid  $800 \text{ mg}$ ; meso-inositol  $800 \text{ mg}$ ; nicotinic  $300 \text{ mg}$ ; Ca-pantothenate  $600 \text{ mg}$ ; pyridoxine HCl  $100 \text{ mg}$ ; riboflavin  $120 \text{ mg}$ ; thiamin HCl  $50 \text{ mg}$ ; folic acid  $13 \text{ mg}$ ; cyanocobalamin  $1 \text{ mg}$ ; menadione bisulfite  $60 \text{ mg}$ ; choline chloride  $2,000 \text{ mg}$ .

¶ Ash, protein and lipid content was calculated based on dry matter.

TABLE 2.

Levels of  $\alpha$ -tocopherol acetate supplemented to the experimental diets

Variable Ingredients	Diets			
	E1	E2	E3	E4
Carboxymethylcellulose ( $\text{mg kg}^{-1}$ diet)	1000	800	400	0
$\alpha$ -tocopherol acetate ( $\text{mg kg}^{-1}$ diet)‡	0	200	600	1000
$\alpha$ -tocopherol equivalent ( $\text{mg kg}^{-1}$ diet)	0	100	300	500

\* ROVIMIX E-50 (Roche Sunve Vitamins Ltd., Shanghai), containing  $500 \text{ mg } \alpha$ -tocopherol  $\text{g}^{-1}$ .

During the postablation period, the remaining females were examined daily and those with mature ovaries were transferred to tanks containing the corresponding group of males to mate. Each mature female with an attached spermatophore was placed into an individual 120-L spawning tank. In the next morning, spawns were examined and shrimp were returned to their respective maturation tanks. Fecundity (number of eggs per spawn) was estimated by counting three 50-mL samples of water in the spawning tank after vigorous mixing to ensure a homogenous suspension of eggs. Fertilization rate, based on the presence of a double membrane and/or embryonic development in eggs, was determined from examination of three 50-mL samples from each spawn. A sample of 10,000 eggs from each spawn was individually incubated at  $29^\circ\text{C}$  to estimate the hatching rate (% nauplii/fertilized eggs). Hatching rate was calculated by counting the number of nauplii per spawn sample through collection by positive phototropism. Egg diameter was estimated by measuring 50 eggs from each spawn with a light microscope and micrometer. About 100 mg of eggs were sieved from each spawn and immediately rinsed in freshwater and stored at  $-70^\circ\text{C}$ . Egg samples from the same group of females were pooled to obtain sufficient tissue for  $\alpha$ -tocopherol analysis. At the end of the experiment, five stage II females from each group were dissected. Hepatopancreata, ovaries and muscle of females from each dietary treatment were pooled and stored at  $-70^\circ\text{C}$  for latter  $\alpha$ -tocopherol analysis. Spermatophore quality was based on sperm count and spermatophore weight according to Alfaro & Lozano (1993). At the end of the experimental period, five males with mature spermatophores from each treatment group were selected, and their spermatophores were extruded by gently pressing around the coxae of the fifth pair of pereopods. Both sides of the compound spermatophore were weighed to the nearest 1 mg. The quantities of sperm were determined by homogenizing the compound spermatophore in a calcium-free solution and by counting the sperm cells under light microscopy.

#### Biochemical Analysis

Triplicate biochemical analysis of the basal diet was conducted according to the following standard procedures (AOAC 1990). Moisture was determined by oven drying to constant weight at  $105^\circ\text{C}$ . Crude protein ( $\text{N} \times 6.25$ ) was derived from Kjeldahl nitrogen analysis. Ash was determined as the residue after muffle furnace ignition at  $550^\circ\text{C}$  for 6 h. Total lipid content was determined after Soxhlet extraction with petroleum ether at  $60^\circ\text{C}$  for 8 h.

Tissue  $\alpha$ -tocopherol content was determined triplicately with HPLC (HP 1100) using methods modified slightly from those of Alava et al. (1993) and Huo et al. (1999). The analytical condition of HPLC was as follows: detection at UV-284 nm; column tem-

perature, 35 °C; eluent, methanol-water (96: 4, v/v); flow speed, 1.0 mL/min.  $\alpha$ -tocopherol was extracted from samples by the method of Huo et al. (1996). Alpha-tocopherol was extracted from 0.2 g of freeze-dried samples by homogenization in 2 mL of methanol containing butylhydroxytoluene (1 mg/mL). The homogenates were clarified by centrifugation and passed through a 0.45  $\mu$ m-pore-size syringe filter (SIGMA). Twenty microliters of the filtrate were introduced into the HPLC injection port. Alpha-tocopherol (95%, SIGMA) was used as standard.

#### Statistical Analyses

An individually marked female within the same treatment group was considered to be an experimental unit for statistical analysis. This is the most commonly used statistical procedure applied in shrimp reproduction trials (Wouters et al. 2002). Data of GSI, HSI, fecundity, daily spawns per female, egg diameter, fertilization rate and hatching rate from each group were subjected to 1-way ANOVA and subsequent Duncan's multiple-range test to determine difference in means. Prior to analysis, Levene's test for homogeneity of variances was used to verify the assumptions for further analysis. There was no need to transform data. A regression analysis was used to determine if a relationship existed between either fecundity, egg diameter, fertilization rate or hatching rate and the spawn order. No relationships were detected, and spawn order was not considered to be a factor to include in the evaluation. An alpha level for all tests was set at 0.05. Statistical analysis was performed using Systat package (Systat Software Inc. 1996).

### RESULTS

Average initial weight of female and male broodstock was  $51.4 \pm 3.56$  g and  $48.02 \pm 5.39$  g, respectively. Survival of male and female broodstock within the different groups is presented in Table 3. A slight loss, probably induced by manipulation, occurred in all groups during the experiment; therefore, number of spawns was calculated based on per female per day.

Alpha-tocopherol concentrations in hepatopancreata, ovaries, eggs and muscle of female *L. vannamei*, as well as in the experimental diets are presented in Table 4. The actual  $\alpha$ -tocopherol concentrations in the experimental diets were higher than the supplemented amounts because the ingredients of the basal diet contained  $37.82 \text{ mg kg}^{-1}$   $\alpha$ -tocopherol. In the present study, female shrimp sampled to determine  $\alpha$ -tocopherol content were at

maturation stage II, with mean GSI values of  $1.45 \pm 0.07$  for group E1,  $1.52 \pm 0.05$  for group E2,  $1.60 \pm 0.04$  for group E3 and  $1.63 \pm 0.07$  for group E4, respectively. Results showed that the  $\alpha$ -tocopherol content of tissue was significantly affected by dietary  $\alpha$ -tocopherol levels. Concentrations of  $\alpha$ -tocopherol in the ovaries and eggs were higher than those in the hepatopancreata. Levels of  $\alpha$ -tocopherol in the ovaries of shrimp in groups E3 and E4 ( $186.43 \text{ mg kg}^{-1}$  and  $207.37 \text{ mg kg}^{-1}$ ) were significantly higher than those of shrimp in groups E2 ( $93.77 \text{ mg kg}^{-1}$ ) and E1 ( $75.04 \text{ mg kg}^{-1}$ ). Alpha-tocopherol concentrations in the hepatopancreata and eggs increased significantly as levels of dietary  $\alpha$ -tocopherol increased, and generally values for each of the groups differed significantly from one another. Supplementation of  $\alpha$ -tocopherol acetate also increased the  $\alpha$ -tocopherol content in muscle. The  $\alpha$ -tocopherol concentration in muscle of shrimp from group E2 ( $50.6 \text{ mg kg}^{-1}$ ) was significantly higher than that of shrimp from group E1 ( $24.5 \text{ mg kg}^{-1}$ ), but was significantly lower than that of either group E3 ( $89.3 \text{ mg kg}^{-1}$ ) or group E4 ( $104.6 \text{ mg kg}^{-1}$ ).

Ovarian maturation and reproductive performance of shrimp representing the different treatments are presented in Table 3. For male broodstock *L. vannamei*, spermatophore weight and sperm count were not significantly different. As dietary  $\alpha$ -tocopherol levels increased, GSI and HSI values of female broodstock did not differ significantly. Average daily spawns per female were significantly affected by dietary  $\alpha$ -tocopherol levels. Females in treatment E3 gave the highest daily spawns, significantly higher than those in treatments E2 and E1. Daily spawns of females in treatments E3 and E4 were not significantly different.

Fecundity and egg diameter were not significantly different among the dietary treatments. Supplementation of  $\alpha$ -tocopherol acetate to the basal diet significantly increased the fertilization rates for treatments E3 (59.6%) and group E4 (61.45%) relative to treatments E2 (51.3%) and E1 (45.4%). The hatching rates for treatments E1 (31.2%) and E2 (34.2%) were significantly lower than those for treatments E3 and E4.

### DISCUSSION

The current study showed that  $\alpha$ -tocopherol content in the basal diet ( $37.82 \text{ mg kg}^{-1}$ ) was sufficient to maintain normal survival, successive maturation and spawns of *L. vannamei*; however, to achieve significantly higher daily spawns, fertilization rates and

TABLE 3.

Maturation and reproductive performance of broodstock *L. vannamei* fed diets supplemented with different levels of  $\alpha$ -tocopherol. Values are mean  $\pm$  S.D. (n), except for survival.

Dietary Treatment	E1	E2	E3	E4
Survival (females)	90%	80%	90%	90%
Survival (males)	86.7%	93.3%	80%	86.7%
Spermatophore weight (g)	$0.066 \pm 0.019$ (5)	$0.071 \pm 0.020$ (5)	$0.075 \pm 0.021$ (5)	$0.073 \pm 0.024$ (5)
Sperm count ( $\times 10^6$ )	$12.43 \pm 4.94$ (5)	$14.16 \pm 5.36$ (5)	$13.22 \pm 4.27$ (5)	$14.53 \pm 4.11$ (5)
GSI <sup>1</sup>	$2.36 \pm 0.81$ (5)	$2.22 \pm 0.44$ (5)	$2.52 \pm 0.48$ (5)	$3.12 \pm 0.77$ (5)
HSI <sup>2</sup>	$3.20 \pm 0.57$ (5)	$3.37 \pm 0.45$ (5)	$3.62 \pm 0.74$ (5)	$3.60 \pm 0.50$ (5)
Daily spawns per female*	$0.030 \pm 0.013^a$ (7)	$0.042 \pm 0.015^b$ (8)	$0.074 \pm 0.030^c$ (9)	$0.071 \pm 0.021^c$ (9)
Fecundity ( $\times 10^3$ )	$128.84 \pm 20.96$ (10)	$130.15 \pm 22.80$ (15)	$130.91 \pm 29.26$ (29)	$123.25 \pm 31.17$ (29)
Egg diameter ( $\mu$ m)	$266.54 \pm 6.21$ (50)	$268.31 \pm 5.76$ (50)	$265.87 \pm 4.30$ (50)	$267.83 \pm 2.34$ (50)
Fertilization rate (%)*	$45.37 \pm 4.17^a$ (10)	$51.26 \pm 5.96^a$ (15)	$59.59 \pm 7.05^b$ (29)	$61.09 \pm 4.25^b$ (29)
Hatching rate (%)*	$31.15 \pm 2.76^a$ (10)	$34.22 \pm 6.22^a$ (15)	$55.20 \pm 6.25^b$ (29)	$53.15 \pm 10.69^b$ (29)

\* Values in the same row with different superscripts are significantly different ( $P < 0.05$ ).

<sup>1</sup> GSI, gonadosomatic index; <sup>2</sup> HSI, hepatosomatic index.

TABLE 4.

$\alpha$ -tocopherol concentrations (mg kg<sup>-1</sup>) in experimental diets, and in eggs, ovaries, hepatopancreata and muscle of female *L. vannamei* fed the different diets

Dietary Treatment	E1	E2	E3	E4
Experimental diets	37.82 $\pm$ 1.45	115.64 $\pm$ 3.82	349.42 $\pm$ 5.83	522.56 $\pm$ 10.41
Hepatopancreas	10.00 $\pm$ 1.24 <sup>a</sup>	47.90 $\pm$ 6.53 <sup>b</sup>	112.65 $\pm$ 6.56 <sup>c</sup>	156.65 $\pm$ 4.43 <sup>d</sup>
Ovary	75.04 $\pm$ 5.31 <sup>a</sup>	93.77 $\pm$ 3.69 <sup>a</sup>	186.43 $\pm$ 7.14 <sup>b</sup>	207.37 $\pm$ 7.48 <sup>b</sup>
Egg	56.60 $\pm$ 1.24 <sup>a</sup>	163.80 $\pm$ 7.2 <sup>b</sup>	280.42 $\pm$ 7.00 <sup>c</sup>	391.01 $\pm$ 5.34 <sup>d</sup>
Muscle	24.51 $\pm$ 2.9 <sup>a</sup>	50.58 $\pm$ 1.86 <sup>b</sup>	89.27 $\pm$ 7.6 <sup>c</sup>	104.55 $\pm$ 4.6 <sup>c</sup>

$\alpha$ -tocopherol concentrations were based on dry weight. Values are means  $\pm$  SD ( $n = 3$ ). Within the same row, values with different superscripts are significantly different ( $P < 0.05$ ).

hatching rates, supplementation of  $\alpha$ -tocopherol to broodstock diets is essential.

The increase in  $\alpha$ -tocopherol concentrations in eggs, hepatopancreata and ovaries of female *L. vannamei* as dietary  $\alpha$ -tocopherol levels increased was similar to the results obtained by Cahu et al. (1995). In their study,  $\alpha$ -tocopherol concentration in eggs of *Fen. indicus* increased from 177 mg kg<sup>-1</sup> to 587 mg kg<sup>-1</sup> as dietary  $\alpha$ -tocopherol levels increased from 82 mg kg<sup>-1</sup> to 354 mg kg<sup>-1</sup>. The beneficial action of  $\alpha$ -tocopherol is most probably related to its antioxidant properties. The  $\alpha$ -tocopherol accumulated in the egg yolk acts as a natural antioxidant (Harrison 1997), and may be especially important for the embryonic and larval development. This positive effect has been verified in the study of Cahu et al. (1995), who showed that hatching rate of *Fen. indicus* increased significantly, from 28% to 55%, when egg  $\alpha$ -tocopherol concentration increased from 177 mg kg<sup>-1</sup> to 587 mg kg<sup>-1</sup>. In the present study, elevation of  $\alpha$ -tocopherol content in ovaries and eggs relative to dietary supplementation might also suggest its positive effect on ovarian maturation and embryonic development. Wouters et al. (2001) found that  $\alpha$ -tocopherol concentrations in ovaries of wild *L. vannamei* increased as sexual maturation proceeded, and suggested that this vitamin has a critical role in advancing the shrimp maturation process. Similarly, Cavalli et al. (2001) detected a sharp increase of  $\alpha$ -tocopherol in the ovaries of wild *Macrobrachium rosenbergii* during the initial gonadal development from stage I to III. They also suggested the dietary importance of this vitamin to serve as a major antioxidant agent during ovarian maturation. For penaeid shrimp, hepatopancreas and muscle tissues are the main storage organs for  $\alpha$ -tocopherol (Cahu et al. 1995). The lower concentration assayed in muscle of females of groups E1 and E2 might indicate that this vitamin was transported from muscle to ovary during vitellogenesis, as was reported in *Fen. indicus* (Cahu et al. 1995) and *Mar. japonicus* (Alava et al. 1993).

Little information on the effect of broodstock nutrition on reproductive performance of male broodstock shrimp is available. In the present study, lack of a significant change in spermatophore weight and sperm count as levels of dietary  $\alpha$ -tocopherol increased

suggests that further studies need to be conducted to evaluate the effect of higher dietary  $\alpha$ -tocopherol levels on the spermatophore quality of male broodstock *L. vannamei*. GSI and HSI indicate the accumulation of nutrients in the ovaries and hepatopancreata of female *L. vannamei*. Similar to a previous study conducted by Du et al. (2004b), the period between eyestalk ablation and the first maturation of females with intact telsons was only 6 days, and the similar values of GSI and HSI might be attributed to the relatively short period of time. In the present study, females fed diets containing 349.42 and 522.56 mg kg<sup>-1</sup>  $\alpha$ -tocopherol gave significantly higher daily spawns, suggesting that at least 349.42 mg kg<sup>-1</sup> of  $\alpha$ -tocopherol in broodstock diet was needed to significantly enhance the ovarian maturation of female *L. vannamei*. Alava et al. (1993) also demonstrated the positive effect of  $\alpha$ -tocopherol on ovarian maturation, and showed that 482 mg kg<sup>-1</sup>  $\alpha$ -tocopherol in the broodstock diet was necessary to cause ovarian maturation of *Mar. japonicus*.

Alpha-tocopherol is also an important factor to improve the fertilization and hatching performance of shrimp eggs. In the study conducted by Cahu et al. (1995), hatching rate increased significantly, from 28% to 55%, when dietary  $\alpha$ -tocopherol levels increased from 82 mg kg<sup>-1</sup> to 354 mg kg<sup>-1</sup>. The positive effect of high dietary  $\alpha$ -tocopherol content on the improvement of egg hatchability was concluded. Similarly, egg hatching rate was also positively correlated with  $\alpha$ -tocopherol concentration of eggs in the studies conducted by Cahu et al. (1991, 1993). Results of this study showed that diets containing  $\alpha$ -tocopherol levels of at least 349.42 mg kg<sup>-1</sup> and fed to *L. vannamei* broodstock could significantly increase the fertilization rate and hatching rate of eggs.

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## INFERRING SHRIMP (*PANDALUS BOREALIS*) GROWTH CHARACTERISTICS FROM LIFE HISTORY STAGE STRUCTURE ANALYSIS

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**ABSTRACT** An aged-based population model was used to simulate shrimp (*Pandalus borealis* Krøyer) age and stage structures at different growth rates and the results compared with stage structures of populations with known growth rates. Both simulations and field data indicate that different growth rates result in characteristic stage structures, which can be used to infer spatial and temporal differences in growth rates. It is concluded that the mean carapace lengths of all life history stages ordered chronologically (stage structure), contains more information on growth characteristics than the carapace length of individual stages (e.g., length at sex change,  $L_{50}$ ) alone. In the absence of growth information, based on direct ageing methods, it is recommended that the complete stage structure be used when inferring growth characteristics from carapace length data. In addition to allowing specific spatial and temporal comparisons of growth rates, stage structure analysis allowed inference of the following growth characteristics, including: growth rates are highly site specific and sensitive to environmental conditions such as food availability and temperatures; environmental influences on growth rates tend to affect all size categories (i.e., stages and ages) simultaneously and without time lags; the “invariant” relationship between  $L_{50}$  and maximum size ( $L_{max}$ ) is not unique to these stages and simply a consequence of environmental influences on the growth rate of all stages simultaneously; length at sex change is directly related to growth/metabolic rates; length and age at sex change are highly flexible, occurring at the age and size a shrimp happens to be when growth/metabolic rates determine sex change. The usefulness of stage structure analysis as an indicator of ecosystem change is discussed in light of the results.

**KEY WORDS:** northern shrimp, *Pandalus borealis*, growth, sex change, life history stage, ecosystem indicators

### INTRODUCTION

Measuring the growth of crustaceans is limited by their lack of hard parts that could be used to directly determine age with confidence. Length-based methods, such as modal analysis (e.g., MacDonald & Pitcher 1979), can be used to determine length-at-age of crustaceans *in lieu* of more direct determinations. However, these methods are fraught with inaccuracies, primarily because modes become more difficult to distinguish and associate with a particular age or year class as age and length increase. In northern shrimp *Pandalus borealis* (hereafter also called shrimp), longevity can vary from 4–8 y or more depending on locality and time (Shumway et al. 1985, Bergström 2000), yet for many stocks only ages 2 and 3 are most consistently identifiable as distinct modes in length frequencies. This has seriously limited the development of quantitative, age-based assessment methods and research on spatial or temporal variations in growth rates (Frechette & Parsons 1982).

Carapace lengths at sex transition, and other life history stages, have been used to infer growth characteristics for *P. borealis*. For example, Koeller et al. (2003) concluded that on the Scotian Shelf the size at which these protandric hermaphrodites change sex from male to female ( $L_{50}$ ), and their maximum size ( $L_{max}$ ), are largely determined by growth rate. Wieland (2004) and Koeller et al. (2006) used size at sex transition and of other stages to infer temporal changes in growth rate off West Greenland and on the Newfoundland-Labrador Shelf. Older age classes are seldom identifiable by distinct modes, but they can often be differentiated in length-frequency analyses by separating female life history stages. Thus, distinguishing between primiparous females (about to spawn for the first time) and multiparous females (spawned before) using the presence or absence of sternal spines often allows identification of two female age classes (Frechette & Parsons 1982). Although the youngest age classes may be distinguishable by the presence of distinct modes, and the oldest by sternal spines, intermediate male year classes immediately before sex change are often

indistinguishable such that it is difficult to relate size at sex change to growth rate. Consequently, an increase in the length at sex change could be because of an increase or a decrease in growth rate, depending on whether longevity, specifically the number of male age classes has, respectively, remained the same or has increased.

Another problem with relating  $L_{50}$  to growth is the possibility that  $L_{50}$  is determined by factors other than growth, such as sex ratio and social interactions, associated with the “sex allocation theory” of Charnov (1982). Another theory by the same author advocates that the ratio of  $L_{50}/L_{max}$  in *P. borealis* is a biological invariant (Charnov 1993, Charnov & Skúladóttir 2000) that provides insight into the evolutionary mechanisms behind sex change in a wide variety of species. In addition to being somewhat contradictory with the former (Koeller et al. 2003), the latter theory has recently come under attack for fundamental analytical reasons (Cipriani & Collin 2005, Nee et al. 2005, de Jong 2005).

This paper takes a more comprehensive look at the use of shrimp life-history stage length statistics in the inference of growth characteristics and discusses the results in relation to current theories on the factors controlling sex change. The approach taken is to compare different growth rates and the “stage structures” they generate using an age-based population model, and to compare the simulated stage structures with those from populations having known growth characteristics. The main hypothesis is that the shape of the curve created by joining the means of life-history stage carapace lengths ordered chronologically (i.e., the stage structure as opposed to the age structure of the population) can provide more information on growth characteristics and other aspects of shrimp biology than that produced when stages are considered in isolation, or in combination with only one other stage, such as the ratio of  $L_{50}/L_{max}$ .

### METHODS

#### Calculation of Stage Statistics

Note that the term “stage length” used throughout refers to the mean carapace length of that stage and not the stage duration. The

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mean lengths of six life history stages were included in the analysis:

$L_{\min}$ —the carapace length of the smallest shrimp in a sample, averaged over a number of samples. This is the size at which shrimp first assume a more benthic life style and begin to be retained by bottom trawls. This metric must be strongly influenced by mesh size and other gear selectivity factors. However, under certain conditions  $L_{\min}$  may also be indicative of growth between hatching and the time of capture. An important condition is that the gear captures some of the smallest (i.e., in the youngest year class) individuals in the population and that the subsample (of the total catch) size is large enough to measure at least one of these. Shrimp trawls usually do capture (and samples show the presence of some, although not many) very small individuals (<10 mm). On the Scotian Shelf, shrimp caught with special juvenile sampling gear in the June survey are ~1.25 y old (~8 mm average carapace length) and have been on the bottom for at least 8 mo (Koeller et al. 2005). The length distribution is normal with a standard deviation of ~1.5, consequently the largest members of the first year class will be several millimeters longer than their mean size, and should be present in many trawl samples. There is usually little or no overlap between the lengths of ages 1 and 2 (mean size ~14 mm), so it is likely that shrimp <10 mm are from the first year class, at least on the Scotian Shelf. Two other conditions that are necessary to relate  $L_{\min}$  to growth for comparison between areas and times is that selectivity should not change, and that samples should be collected at the same time of year. These conditions are usually met within a survey series, but not between series using different gear types, cod-end mesh sizes or survey times, or for samples collected from commercial fishery catches, which are obtained from different vessel and trawl gear types.

$L_m$ —the average size of males in a sample, averaged over a number of samples. In addition to growth, this statistic will be influenced by other population dynamics including recruitment and mortality. For example, in a population of four male age classes where the three youngest had average recruitment and the oldest strong recruitment,  $L_m$  will be larger than if recruitment had been the same for all year classes. Note that shrimp generally mature as males when quite small—usually (and in this paper) no distinction is made between mature and immature males.

$L_{50}$ —the length at which half of the shrimp in a sample are female, averaged over a number of samples. This statistic was estimated by fitting a logistic model to cumulative percent (female) length-frequencies of individual samples and estimating the length at which 50% of the shrimp were female as described in Skúladóttir (1998). All model fitting was carried out using the *glm* function in S-PLUS Version 6.2 (Insightful Corp.) with the logit function link to the equation:

$$y = 1/(1 + e^{-(a+bx)})$$

where  $y$  is the estimated proportion of females,  $x$  is the carapace length,  $a$  is the regression coefficient and  $b$  is the steepness coefficient of the logistic curve. This statistic can also be influenced by changes in population dynamics. All other factors like growth rate being equal,  $L_{50}$  will be larger if the last male year class is more abundant than the others. It will decrease if the population of first year (primiparous) females is larger, or their mortality is less than average. Sampling time, or annual variability in the time and speed at which sex change in the population occurs may also affect this statistic, for example, if a larger percentage of males than usual have changed sex before the survey period then the average size at

sex change will be lower, and vice-versa. The methods of calculation used by different workers may lead to different results depending on which component of the female population is used to calculate the percentage of females. In this paper it is calculated using primiparous females wherever possible, because it is these females that are changing sex during the sampling year. However, in Newfoundland the survey is conducted in November-December at a time when only a small number of, or no, primiparous females, which have not become ovigerous are present. In this area  $L_{50}$  was calculated from the percentage of total females. In addition to the above factors,  $L_{50}$  may also be influenced by seasonal sampling times. However, variation because of this factor are minimized by identifying transitional (undergoing sex change) individuals and including them in the female category when sampling occurs during the relatively short (several months) period when shrimp are actively changing sex.

$L_{f1}$ —the average size of primiparous females in a sample, averaged over a number of samples. Primiparous females are identified as females with sternal spines (McCrary 1971). These spines are located in the abdominal area where eggs are brooded and tend to be lost when the female first extrudes eggs. A female with sternal spines has therefore not spawned before and probably belongs to the youngest female age class. In this paper  $L_{f1}$  includes shrimp that have been identified as "transitional" (i.e., in the process of changing sex) because this stage is relatively short and all shrimp in this category will join the primiparous population that year.

$L_{f2}$ —the average size of multiparous females in the sample, averaged over a number of samples. Multiparous females are identified as females without sternal spines, and so have spawned before. Because natural mortality is high after two years as females, most females in this category will usually be second year females. However, a certain number of older females are likely present, especially if the length-frequency distribution of this stage is skewed to the right. Note that the time of sampling within the annual spawning cycle determines if  $L_{f1}$  and  $L_{f2}$  can be distinguished from each other. Because most  $L_{f1}$  females will have spawned by fall, a survey at this time will usually not be able to distinguish these stages (e.g., the Newfoundland survey series). Moreover, commercial samples in this study do not distinguish between the two stages of females even if both are present. In these cases mean female size is calculated from all females combined and is designated as  $L_f$ .

$L_{\max}$ —the largest shrimp in a sample, averaged over a number of samples. This can be taken as a proxy for  $L_x$  in the Von Bertalanffy growth equation. Unlike  $L_{\min}$ , selectivity should have little effect on this parameter, because most shrimp in this size range are likely to be retained by the fishing gear. However, as with  $L_{\min}$ , sample size (number of shrimp per sample) may be a factor. For example, a heavily-fished population will have fewer of the largest shrimp. Although some will still be present in the population, a larger sample size may be required to ensure they are also present in the sample. The largest shrimp are likely older than the second female year class, particularly if the female length frequency is right-skewed.

### Simulations

A simple age-based model was used to simulate shrimp growth and the population length, age and stage structure for the areas under consideration, using a range of growth rates and longevities

encompassing those reported in the literature for the areas under consideration (Table 1). The number of male age classes was varied between 2 and 5, which encompasses the number of male age classes in the Gulf of Maine (2–3), the Scotian Shelf (3–4) and the southern Newfoundland Shelf (4–5). It was assumed that all populations had only two female age classes corresponding to the common primiparous and multiparous designations ( $L_{T1}$  and  $L_{T2}$  above). Although there may be more than two female age classes present in the population, in general mortality is considered to be high after the second year as females and relatively few survive a third year. The length structure for each age class was generated with a normal distribution function using parametric standard deviations (sigmas) similar to those seen in shrimp modal analyses with the MIX computer program (Macdonald & Pitcher 1979). Sigmas ranged between 1 and 1.6 and were increased by increments of 0.1 units/year with increasing age, held constant for all simulations. Modal lengths of age classes (i.e., growth) were determined by a von Bertalanffy growth function using parameters determined from available length at age data for specific stocks. Generally, lengths at age and growth for *P. borealis* stocks are determined with modal analysis. This is best done by an experienced analyst having local knowledge of the stock. Lengths-at-age information for the three stocks (Table 1) examined here were therefore obtained from stock assessment documents or other sources as follows: Gulf of Maine, Anon. (2004); Scotian Shelf, Koeller et al. (2005); Newfoundland Labrador Shelf, Orr et al. (2003); Parsons et al. (1986, 1989). Year-class strengths were held constant and equal for all age classes unless the sensitivity to changes in year-class strength was being tested. Selectivities were determined by a logistic curve using the same equation described under  $L_{50}$  above, but where  $y$  is the selectivity factor,  $a = 0.5$  and  $b = 15$ . This provided a typical sigmoid cumulative selectivity curve resulting in a 50% retention length of 15 mm with a length range of 4.4–30 mm. Because it was assumed that all ages and stages were represented in the population (sample), variations of the selectivity function, while changing the relative abundance of ages and stages considerably, had little effect on average age and stage lengths. Consequently selectivity was held constant for all simulations. The total mortality factor (constant for all simulations) was set at a survival rate of 0.5 ( $Z = 0.70$ ) (i.e., half of the population died each year until the second year as females) when mortality was total. This is considered a reasonable rate of natural mortality ( $M$ ) for this species in stock assessments and was used as

total mortality, because fishing mortality or other impacts other than selectivity were not simulated. The above-mentioned combination of selectivity and mortality resulted in a typical survey length frequency distribution. Sex change was assumed to occur within one year with all males from the last male year class changing to primiparous females. Stage statistics (mean lengths for  $L_{min}$ ,  $L_m$ ,  $L_{50}$ ,  $L_{T1}$ ,  $L_{T2}$  and  $L_{max}$ ) were then calculated as population parameters (i.e., sampling was not simulated).

#### Survey Data

Length statistics were examined from three stratified random shrimp survey series used in the stock assessments of three distinct stocks, including the Gulf of Maine (Anon 2004), the eastern Scotian Shelf (Koeller et al. 2005) and the Newfoundland Shelf (Orr et al. 2003). Survey methods, which are provided in the publications above, differ somewhat between these regions, but within each series all use standardized survey tow lengths and shrimp trawls. Within each series, surveys are conducted at the same time of year. All surveys use the same carapace length measurement technique (oblique length from the posterior edge of the eye socket to the posterior middorsal edge of the carapace) and criteria for distinguishing life history stages (i.e., between males and females, Allen 1959) and between primiparous and multiparous females (McCrary 1971). An important difference between methodologies is survey time—the Gulf of Maine and the Scotian Shelf surveys are conducted during spring or early summer (May to June), whereas Newfoundland surveys are conducted during fall winter (November–January). Because the population's stage structure changes seasonally, this must be taken into consideration when comparing stage structures between areas, for example the lack of primiparous females in the Newfoundland series described earlier. Because some survey catches can be small and sample carapace lengths inaccurate only samples with at least 200 shrimp, including both males and females, were included in the analysis. After filtering the data according to this criterion a total of about 5,500 survey samples from the three survey series remained available for analysis (Table 2). The locations of the primary areas considered in this paper are shown in Figure 1.

#### Commercial Fisheries Data

In addition to survey samples, a large number (>20,000) of commercial samples were collected between 1990 and 2002 from 47°N (off Newfoundland) to 67°N (Davis Strait). These were collected by trained observers, with onboard analyses similar to survey samples, including carapace length measurements to the nearest 1/10 mm, and determination of life history stage, although the latter was limited to identification of males and females only (i.e., transitionals, primiparous and multiparous females were not distinguished). The large number of samples allowed more detailed analyses of stage-length statistics, particularly the relationship between stages, and between stage size and environmental parameters. The total number of samples that were available for analysis by area, year and data source is given in Table 2.

## RESULTS

#### Growth Simulations

In the first set of simulations (Fig. 2A), growth rates were held constant, but longevity varied from 4–7 y (2–5 male age classes, 2 female age classes). The most notable result is that the shape of

TABLE 1.

Lengths (mm) at age (in years) and parameters from von Bertalanffy growth curves fitted to them. Length and age data sources: Anon 2004, Koeller et al. 2005, Orr et al. 2003, Parsons et al. 1986, 1989.

Age	Gulf of Maine	Scotian Shelf	S. Nfld-Lab. Shelf
1	11.2	9.0	10.0
2	18.4	14.9	14.4
3	23.5	18.0	17.2
4	27.0	20.3	19.5
5		22.2	20.5
6		24.1	22.9
7			24.5
$L_{\infty}$	35.23	26.89	28.61
K	-0.3591	-0.3463	-0.242
$t_0$	-0.06178	-0.01079	-0.21

TABLE 2.

The total number of samples which were available for analysis by area, year and data source. Gulf of Maine, Scotian Shelf and Newfoundland-Labrador surveys were conducted in May, June and October to January, respectively. Commercial samples were collected throughout the year.

	Gulf of Maine Survey	Scotian Shelf Survey	Newfoundland	
			Survey	Commercial
1982	—	30	—	—
1983	17	29	—	—
1984	35	29	—	—
1985	50	25	—	—
1986	45	24	—	—
1987	47	22	—	—
1988	40	31	—	—
1989	47	—	—	—
1990	43	—	—	1910
1991	45	—	—	2336
1992	47	—	—	1316
1993	49	—	—	953
1994	37	—	—	1286
1995	48	48	171	1351
1996	40	57	424	1383
1997	48	55	468	1667
1998	44	59	514	1476
1999	50	57	525	1556
2000	46	58	363	1972
2001	44	65	430	1632
2002	50	57	185	1753
2003	43	64	453	—
2004	—	52	256	—
Total	915	732	3789	20591

the stage structure changed significantly with the number of male age classes. The shortest longevity (lowest number of male age classes) had the straightest stage structure, whereas the longest had the greatest curvature characterized by a rapid increase in length to a peak at  $L_{50}$ , a decrease at the first female stage, followed by an increase to  $L_{max}$ . Intermediate longevities had intermediate degrees of curvature to their stage structures. With growth rates held constant, longer longevities and greater curvatures also have correspondingly larger stage lengths for all stages except  $L_{min}$ . Note that this simulation is intended to be illustrative rather than realistic, because one would expect increased longevities to be associated with decreased growth rates.

In the second simulation (Fig. 2B) longevities were held constant at the 6 age classes typical for many shrimp stocks (4 male, 2 female) but growth rates varied. In these simulations stage structure (curve shape) changed little. All exhibited the rapid increase in length to  $L_{50}$ , decreased rate of increase to the female stages and more rapid increase to  $L_{max}$  noted in the previous simulation. All stage lengths, including  $L_{min}$ , increased with growth rate.

A third set of simulations (Fig. 2C) used typical growth rates and longevities reported for the three study regions (Table 1). The fastest reported growth rate and shortest longevity (only 2 male age classes) is from the Gulf of Maine. The Scotian Shelf simulation used 4 and the Newfoundland-Labrador Shelf 4 and 5 male age classes. Longevities reported for the Newfoundland-Labrador Shelf vary considerably from 4–6 male age classes, with 4 and 5 being the most common (Parsons et al. 1986, 1989). Growth rates

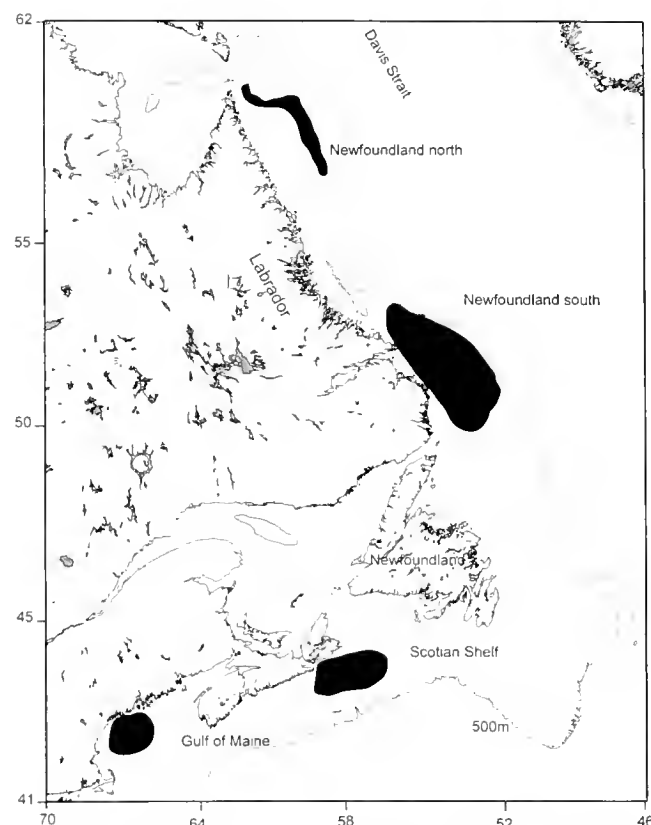


Figure 1. Map showing the 3 main study areas (Gulf of Maine, Scotian Shelf and the Newfoundland-Labrador Shelf). The latter is divided into northern and southern parts.

reported for the Scotian Shelf are somewhat faster than those for the Newfoundland Shelf. As would be expected from Figure 2A, the simulation using the short longevities reported from the Gulf of Maine showed the straightest stage structure. Stage lengths are about the same as for other areas, not smaller as would be expected if growth rates were the same. Apparently the much higher growth rate in the Gulf of Maine compensated for the shorter longevity, particularly in the three oldest stages, which were considerably larger than those in the other areas. Because of its slower growth rate, the Newfoundland-Labrador curve with only four male age classes fell well below that of the Scotian Shelf. When five male year classes are used for the Newfoundland-Labrador Shelf the curves for these two areas are indistinguishable despite their being based on different longevities. The increase in stage lengths expected with increased longevity alone, as in Figure 2A, compensated for the decrease in stage length expected from lower growth rate alone, as in Figure 2B.

A final set of simulations (Fig. 2D) explored the effects of a large recruitment event in which a strong year class ( $100 \times$  average recruitment) moves through the population. Longevities and growth rates were held constant, using values characteristic for the Gulf of Maine, whose shrimp population size and fishery are highly dependent on such events. The effects of the strong year class changed stage structure considerably in the younger stages, including  $L_{50}$ . On the other hand,  $L_{min}$ , which in this case was determined by selectivity alone, was not changed. The appearance of a large number of young males decreased  $L_m$  in Year 1. In Year 2 the large number of males about to change sex increased the

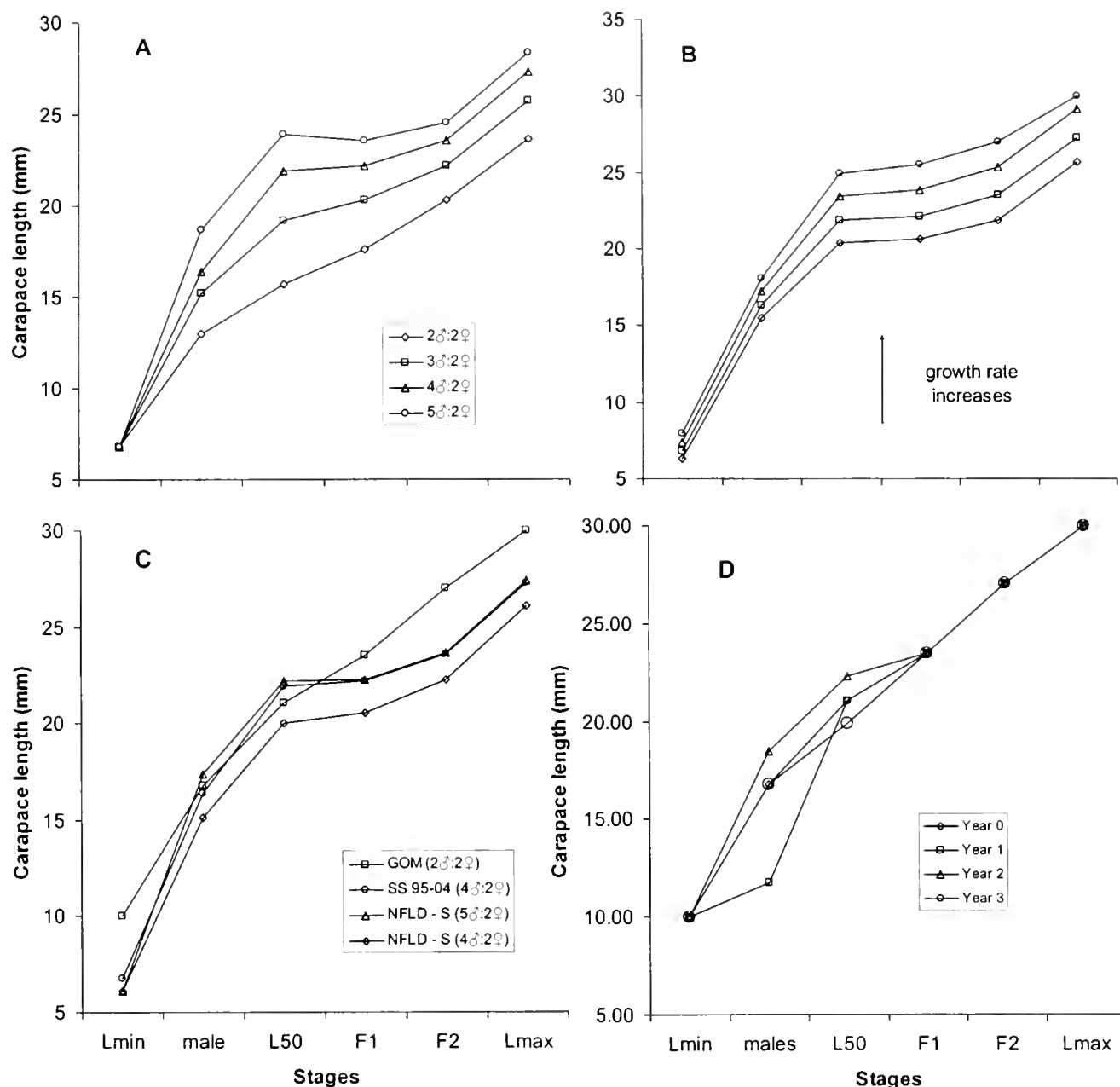


Figure 2. Simulation results including (A) constant growth rates but longevity varying from 2–5 male year classes; (B) constant longevity (4 male and 2 female age classes) but varying growth rates; (C) observed growth rates and longevity for the Gulf of Maine, Scotian Shelf and southern Newfoundland Shelf. The latter includes curves for 4 and 5 male age classes. (D) simulated annual stage lengths where a strong year class ( $100 \times$  average recruitment) appears at Year 1 and moves through the population over the next 2 y. The population has two male and two female age classes as in the Gulf of Maine.

estimate of  $L_{50}$  because more females are needed to reach the 50% point, which are found farther along the ascending limb (at longer carapace lengths) of the female frequency distribution. In Year 3 the strong year class was in its first year as females, and  $L_{50}$  had decreased almost back to its pre-event (Year 0) value. Stage sizes after  $L_{50}$  were unchanged during all years.

#### Survey Data

The age and stage structures for two survey years (1995 and 2000) where both are available in all three areas are shown in Figure 3. Both years show the exceptional growth rate and shorter

longevity usually exhibited by Gulf of Maine shrimp. Despite the large difference in growth rates, shrimp on the Scotian Shelf and the Gulf of Maine change sex at about the same length (~22–23 mm) and achieve similar maximum lengths of 29–30 mm (Fig. 3 C, D). However, Gulf of Maine shrimp change sex after only 2 y as males, whereas on the eastern Scotian Shelf there are usually four male year classes. Growth rates on the southern Newfoundland Shelf are somewhat slower than on the Scotian Shelf, although longevity appears to be similar. Sizes at sex change (~21–22 mm) and maximum size (25 mm) are considerably less on the Newfoundland Shelf compared with those on the Scotian Shelf.

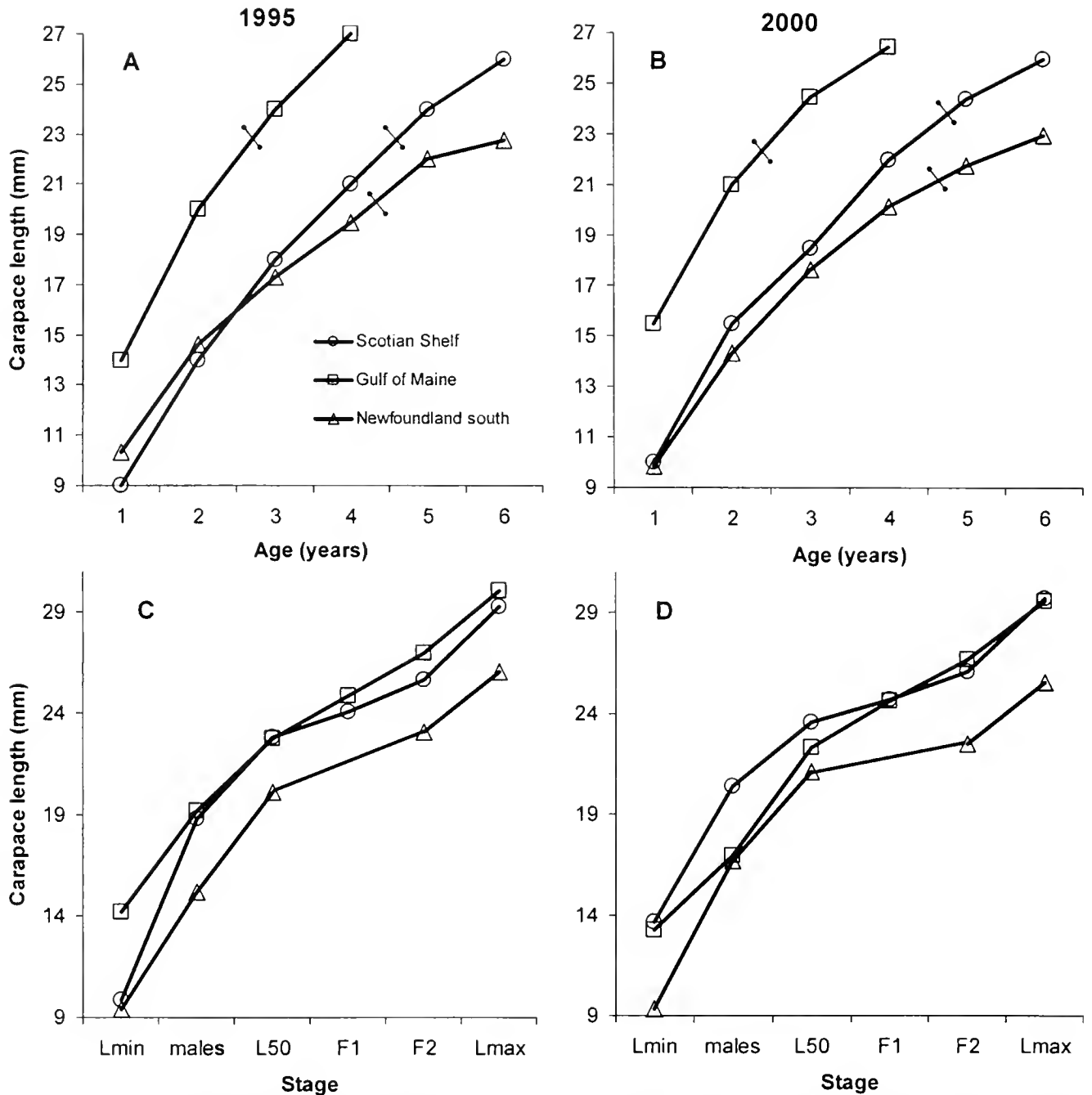


Figure 3. Carapace lengths at age for the Gulf of Maine, Scotian Shelf and southern Newfoundland-Labrador Shelf survey data for 1995 (A) and 2000 (B) illustrating the main differences in growth characteristics between these areas. The sizes and ages at sex change are indicated by diagonal lines. The corresponding stage structures are shown in C and D.

Growth rates are lower (Fig. 3A, B) and stage lengths are shorter (Fig. 3C, D) on the Newfoundland-Labrador Shelf compared with those for Scotian Shelf shrimp during both years. This suggests that longevity for these two areas are the same (i.e., 4 male age classes) because simulations indicate that a longevity of five male year classes on the Newfoundland-Labrador Shelf versus four on the Scotian Shelf should have resulted in similar stage sizes (Fig. 2 C). The large difference in growth rates between the Gulf of Maine and the other areas is not reflected in the younger (than  $L_{11}$ ) stage lengths, which are similar to those on the Scotian

Shelf. This is as expected from the simulation (Fig. 2C) (i.e., the longer longevity on the Scotian Shelf compensated for the slower growth rate) resulting in similar stage sizes. However, the older stages in the Gulf of Maine surveys are also about the same size as on the Scotian Shelf, but these were considerably larger in the simulation. Differences in the shape of the curves defined by survey mean stage sizes in the three areas are consistent with simulations. The Gulf of Maine curves are relatively straight compared with those for the other areas in both years. The consistency of the differences in curve shapes and stage lengths between areas is seen

in Figure 4 where the averages of the annual stage mean lengths are plotted for all survey years since 1995, along with standard deviations.

Figure 5 shows annual differences in stage structures for each of the three areas. The Gulf of Maine (Fig. 5A) has the longest time series of targeted shrimp surveys in the north Atlantic, and exhibited a wide range of stage lengths, 4–6 mm for all stages except  $L_{max}$ , which had a narrower range (2 mm). The shape of the annual stage structures varied widely in this area. Many of the plots for the first 10 years of the series exhibit a greater curvature and longer stage lengths, but some years during this period also have straighter curves and shorter stage lengths than the others (e.g., 1983, 1992). Similarly, many of the annual plots during the last 11 y of the series appear to be straighter than during the previous period, however there is also considerable variability. On the Scotian Shelf (Fig. 5B) the earlier years clearly exhibit a greater curvature and longer stage lengths than the later years, which is opposite to the case for the Gulf of Maine. The Scotian Shelf stage structures more clearly separate into two time periods (i.e., 1982–1988) and 1995–2004.

The Newfoundland-Labrador Shelf survey series is the shortest and has the least consistent spatial coverage of all the three main areas. The time period shown (1996–99, Fig. 5C) includes years when survey data are available in both the northern and southern areas of the shelf and significant decreases in shrimp lengths were observed (Orr et al. 2003, Koeller et al. 2006). In the two early years the curves in the northern area are straighter and all stages, especially  $L_{min}$  and  $L_m$ , much longer than those in the south. The greater curvature during the last two years in the north is associated with shorter stage lengths of all stages, but especially of the largest, oldest stages. These decreased greatly to carapace lengths comparable to (1999), or smaller than (1998) in the south.

Figure 5D shows 4 y extracted from Figure 5A during which

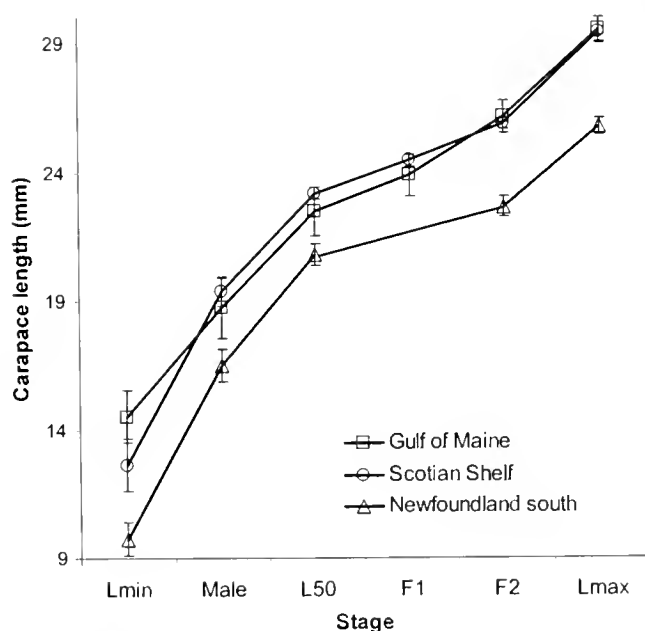


Figure 4. Average stage structures in the Gulf of Maine, Scotian Shelf and Newfoundland-Labrador Shelf surveys for years (1995–2003) when survey information was available for all three areas. Error bars represent  $\pm 1$  standard deviation.

the large 1982 y class moved through the Gulf of Maine population. The actual stage structure is similar to those for the simulated recruitment event shown in Figure 2D, including the similar  $L_{min}$  (except for 1983), progressively older stage lengths becoming larger in sequence, and convergence of the largest stage sizes. However, this convergence occurred at  $L_{12}$  in the surveys versus  $L_{11}$  in the simulations.

#### Commercial Fishery Versus Survey Data

$L_{50}$  carapace lengths are plotted against all other identified stage lengths for all available commercial (20,596) and survey samples (2,158 samples with >200 shrimp/sample) in Figure 6. The slopes of the regressions between  $L_{50}$  (dependant) and other stages are all positive and significantly different from 0. The stage structures around the median and the 1st and 3rd quartile hinges of  $L_{50}$  within each data set are shown in Figure 7. Commercial stage structures are noticeably straighter than those obtained from survey samples. All commercial stages are significantly larger than survey stages, although the difference is considerably less for  $L_{50}$ .

In simple paired correlations the lengths of all stages were usually significantly correlated with all environmental (density, depth), spatial (shrimp fishing areas) and temporal (year and month) factors available for analysis in both data sets i.e., 42 out of 50 (5 factor  $\times$  5 stages  $\times$  2 data sets) correlations significant at  $P < 0.05$ . Depending on the stage used as the dependant variable, all these factors combined accounted for 32% to 73% and 33% to 40% of the variability in stage size for commercial and survey samples, respectively, in linear multiple regression analysis, although most of this was caused by area and year effects (Table 3). In addition to environmental, spatial or temporal factors, stage lengths were also significantly related to each other (Table 3, Fig. 6). Depending on the stage used in multiple regressions, 53% to 78% and 45% to 68% of the variability of any stage was accounted for by the length of the other stages in commercial and survey samples, respectively. The strength of the relationships between stages tended to decrease with increasing differences in length (Table 4). For example, the relationship between  $L_{50}$  in commercial samples was strongest with  $L_m$  and  $L_1$ , the next smallest and next largest stage, respectively, and weaker with the smallest and largest stages (i.e.,  $L_{min}$  and  $L_{max}$ ). Similarly,  $L_{min}$  was correlated most strongly with  $L_m$  and less so for the longer (older) stages. Finally,  $L_{max}$  was most strongly correlated with  $L_1$ , and less so for the smaller (younger) stages, including  $L_{50}$ . Similar results were found for survey data (Table 4).

Table 5 shows the relationship between  $L_{50}$  and  $L_{max}$  as the ratio and its standard deviation, summarized in various ways, including by sample, area, years within areas, and groups of years within areas. The ratios  $L_{50}/L_{max}$  from this study and those summarized by area over a number of years for Icelandic shrimp stocks (Charnov & Skúladóttir 2000) are remarkably similar and not significantly different from each other, in agreement with the observation that this ratio is a biological "invariant" (Charnov 1993, Charnov & Skúladóttir 2000). However, this also holds for the ratio of  $L_m/L_{50}$  and  $L_m/L_{max}$  for the same data groupings in Table 5.

## DISCUSSION

#### Stage Structure and Growth Determinations

The results indicate that stage structure (i.e., the combination of the shape of the line joining the chronologically-arranged mean

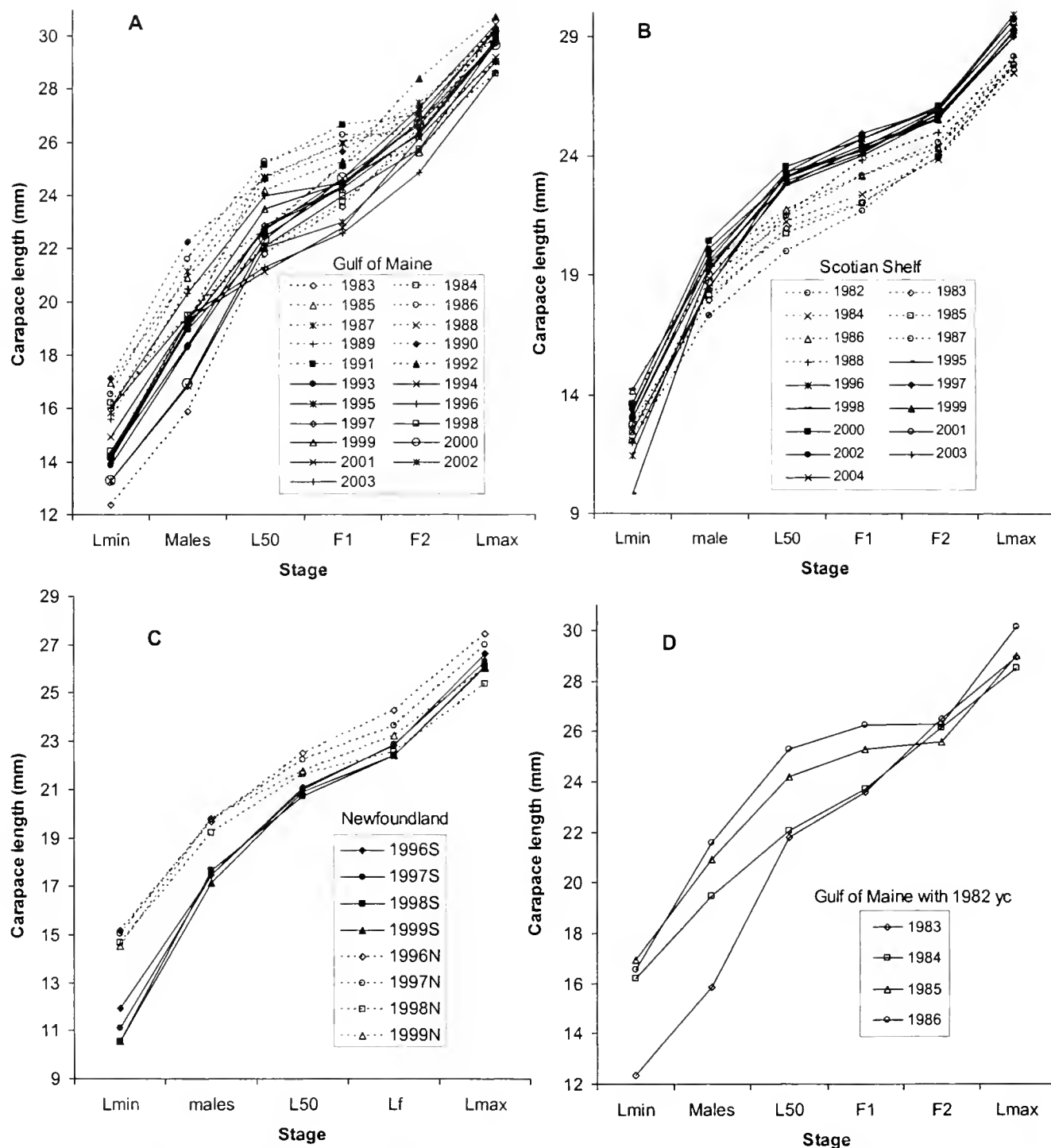


Figure 5. Stage structures for individual survey years in the Gulf of Maine (A), Scotian Shelf (B), northern (N) and southern (S) Newfoundland-Labrador Shelf (C) and the Gulf of Maine survey for 4 y during which a strong year class (1982) was present (D).

stage lengths), and the stage lengths themselves, can provide more information about the growth characteristics of *P. borealis* than the mean lengths of individual stages taken in isolation. For example, both simulations and survey samples showed a straighter stage structure for populations with shorter, and greater curvature for populations with longer, longevity. If longevity does not change over time, as indicated by unchanged curvatures, then an increase or decrease in the lengths of all stages can be taken as an increase

and decrease in growth rate, respectively. Two time periods on the Scotian Shelf previously shown to have different longevity (Koeller et al. 2003) had distinctly different stage structures, including a greater curvature for the period known to have longer longevity. This period also had longer stage lengths, suggesting that the longer longevity was not associated with growth rates low enough to compensate for the associated increase in stage sizes, as seen when comparing Scotian Shelf and Gulf of Maine stage struc-



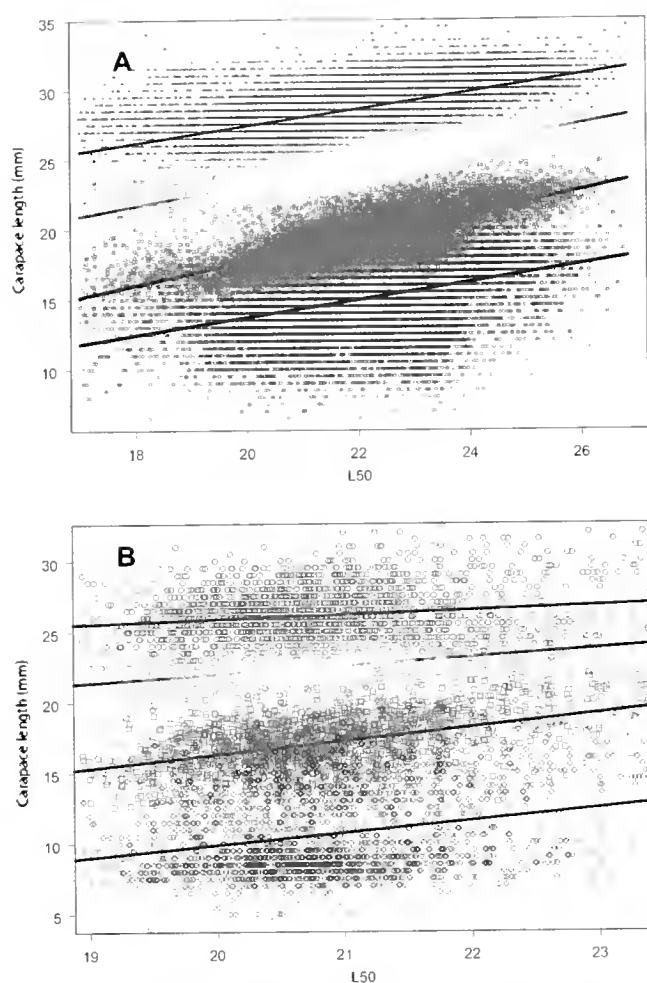


Figure 6. Individual sample estimates of  $L_{50}$  plotted against  $L_{min}$ ,  $L_m$ ,  $L_t$  and  $L_{max}$  in the same sample for (A) all commercial samples and (B) survey samples with >200 shrimp.

tures. The differences in stage structures between areas were remarkably consistent over time and reflected the known differences in growth characteristics between areas. The Gulf of Maine stock has the shortest longevity of those examined and the straightest stage structure. It also has the fastest growth rate, which is reflected by its stage sizes that are the same or larger than the slower growing shrimp on the Scotian Shelf and Newfoundland Shelf. The faster growth rate compensates for the smaller stage sizes expected from shorter male longevity alone. The slower growth rate but similar longevity of the Newfoundland Shelf relative to the Scotian Shelf is reflected in its shorter stages and similar stage structure. A greater longevity, such as has been reported for parts of the Newfoundland Shelf (e.g., Parsons et al. 1989) in combination with slower growth would have resulted in similar stage lengths but greater curvature of the stage structure.

#### An Example Application of Stage Structure Analysis

Koeller et al. (2006) showed that the carapace lengths of most shrimp stages decreased on the entire Newfoundland shelf during the 1990s, with the greatest decrease occurring from 1996–1999 in the northern part of the shelf (Fig. 5C). In addition, shrimp on the northern shelf are generally larger than those in the south. Figure 5C shows that before the large decline in stage sizes (1996–1997)

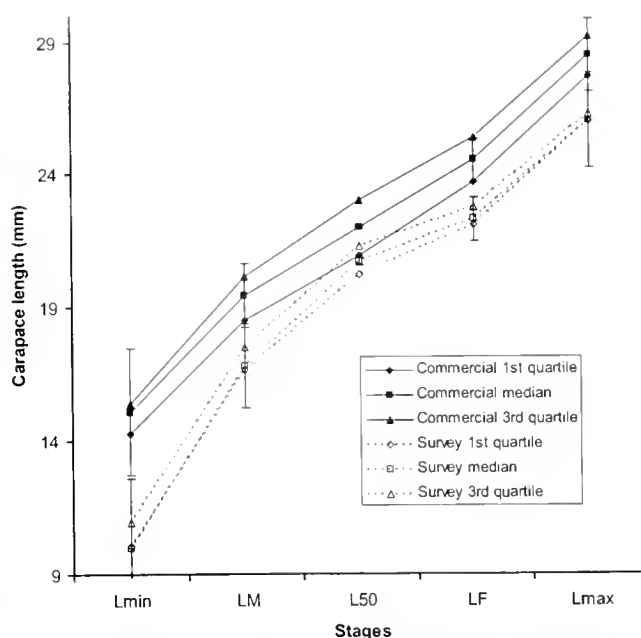


Figure 7. Stage structure for commercial and survey samples at the median and the 1st and 3rd quartile boundaries of  $L_{50}$ . The number of samples used to calculate medians and means around the quartiles are ~1,000 for commercial and ~200 for survey samples. Standard deviations are shown only for the median.

the shape of the stage curves on the northern area are straighter and all stage sizes larger than on the southern Newfoundland Shelf, suggesting that shrimp in the north have both a shorter longevity and faster growth. This is also supported by the larger  $L_{min}$ s in the north—shrimp are much larger before they begin to enter survey nets here, suggesting that they grow very quickly during their first year. The greater curvatures in the north during 1998–1999 may then be because of an increased longevity, but with a concurrent decrease in growth rates because the stages are not larger. These slower growth rates could have resulted from decreased primary

TABLE 3.

Multiple regression R-squares of the average length of each stage versus the length of other stages within each sample. Also shown are R-squares for the length of each stage versus environmental (shrimp density, depth), spatial (Shrimp Fishing Area) and temporal (month, year) factors. Samples are from commercial and scientific surveys collected between 1990 and 2002 on the Newfoundland-Labrador shelf.

Independants		Commercial		Survey	
		Density Depth Area (sfa) Year Month	Other Stages	Density Depth Area (sfa) Year Month	Other Stages
Dependant	$L_{min}$	0.3215	0.5261	0.3601	0.5696
	$L_m$	0.5587	0.7256	0.3997	0.6755
	$L_{50}$	0.6046	0.5852	0.3337	0.4533
	$L_t$	0.7318	0.7848	0.3936	0.6811
	$L_{max}$	0.5242	0.6571	0.4016	0.6322
	$n$	20,587	20,587	2158	2158

TABLE 4.

Pearson correlation coefficient matrix for average stage lengths in all commercial ( $N = 20,591$ ) and survey ( $N = 2158$ ) samples collected on the Newfoundland-Labrador shelf from 1990–2002. Individual commercial values are shown in Figure 6. All coefficients are highly significant at  $p < 0.0001$ . Values for commercial and survey samples are below and above the diagonal, respectively.

		Commercial				
		$L_{min}$	$L_m$	$L_l$	$L_{50}$	$L_{max}$
Survey	$L_{min}$	—	0.8420	0.4758	0.3866	0.4049
	$L_m$	0.7347	—	0.6929	0.7257	0.5776
	$L_l$	0.4412	0.5337	—	0.8236	0.8637
	$L_{50}$	0.2235	0.4469	0.4981	—	0.6084
	$L_{max}$	0.3599	0.4866	0.6839	0.1393	—

production during the late 1990s by the mechanism suggest by Fuentes-Yaco et al. (2006). They hypothesized that the shrimp stages on the northern Newfoundland Shelf are much larger because of faster growth rates from the enhanced primary production at and near the mouth of Hudson Strait (Sutcliffe et al. 1983, Drinkwater & Harding 2001), and that the greater size decrease in this area was because of slower growth from a greater decrease in this enhanced production. The latter could have occurred if the greater amount of freshwater runoff received by the northern Newfoundland Shelf through Hudson Strait during the warming trend of the late 1990s increased water column stratification and decreased nutrient influx into the euphotic zone. This theory is in contrast to the generally accepted explanation of why shrimp in more northern areas are usually larger: that they grow more slowly in colder northern waters, but are larger because they live longer. Limited sampling in the northern areas did not allow satisfactory modal analysis to determine growth rates and longevities more directly. Note that results from the Gulf of Maine also contradict the general observation that faster growth and shorter life spans lead to smaller maximum sizes.  $L_{max}$ , and other stage lengths in this area, appear to be at least as large as in the slower growing northern stocks.

#### Stage Structure and Theories of Sex Change

Stage structure analysis may provide insight into existing theoretical treatments of protandry (Charnov 1982, Charnov 1993, Charnov & Skúladóttir 2000) and the sometimes-conflicting observational evidence supporting them (Bergström 1997, Koeller et

al. 2000, Koeller et al. 2003, Cipriani & Collin 2005, Gardner et al. 2005, de Jong 2005, Nee et al. 2005). The most controversial theory derived from field observations of sex change in *P. borealis* involves the apparent invariance of the ratio  $L_{50}/L_{max}$  off Iceland, which Charnov & Skúladóttir (2000) use to surmise fundamental, mathematically-expressed (in terms of common growth and mortality parameters) rules defining the size at which animals change sex. Nee et al. (2005), de Jong (2005) and Cipriani and Collin (2005) demonstrated that the test for invariance (i.e., a slope of 1 for regressions of log-transformed variable pairs suspected of being invariant) is also positive when bounded variables are generated randomly. However, Cipriani and Collin (2005) also showed that the results of Charnov and Skúladóttir (2000), when expressed as ratios of  $L_{50}/L_{max}$  rather than regression statistics, support the hypothesis that the Icelandic results arose from life-history invariant-related, and not random, processes. The present results suggest that the ratio  $L_{50}/L_{max}$  is indeed invariant for other *P. borealis* stocks and not statistically different from the Icelandic results. However, they also indicate that its status as an invariant is not unique—similarly insignificantly different mean ratios and low standard deviations were obtained for other ratios, including ones without  $L_{50}$  as one of the parameters e.g.,  $L_m/L_{max}$ . Clearly, although the invariance of  $L_{50}/L_{max}$  does not appear to be an “illusion” as suggested by Nee et al. (2005), the contention that it constitutes a fundamental principle in the evolution of sex changing animals should be examined in light of the present results.

Within-sample relationships between  $L_{50}$  and the other stages, including the apparent invariance between stages, manifest themselves statistically only when the average stage lengths of many samples are regressed against each other (Fig. 6A), or when within-sample average stage lengths are averaged over many samples (Table 5). Whereas the relationships between stages are relatively constant or invariant “on average” there is still a large amount of residual variation as shown by the wide scatter around the regression lines in Figure 6. Given that the within-sample relationships between stages are mainly determined by growth processes, which in turn are driven by local food availability and temperature, the “cigar-shaped” edges of the scattergrams in Figure 6 must represent the physiological limits of length and growth for all stages. This is supported by the decreasing scatter at either end of the “cigars.” More constraints and less variation are to be expected near these limits, a situation analogous to the decrease in variation of winning statistics at extreme levels of sports achievements, which are near the physiological limits of the species concerned. Under these circumstances it is not surprising that the

TABLE 5.

Ratios of various stage pairs summarised by sample, area and year, including  $L_{50}/L_{max}$ , the ratio considered to be a biological “invariant” (Charnov & Skúladóttir 2000). There is no significant difference between the means within any set of ratios. All samples were collected from scientific surveys except for Newfoundland commercial samples.

	$L_m/L_{max}$		$L_m/L_{50}$		$L_{50}/L_{max}$		Level of data aggregation	N
	Mean	SD	Mean	SD	Mean	SD		
NFLD commercial	0.68	0.05	0.88	0.05	0.77	0.04	Sample	19705
NFLD survey	0.65	0.06	0.82	0.08	0.80	0.07	Sample	2158
Iceland (Charnov and Skúladóttir 2000)	—	—	—	—	0.80	0.01	Area	21
Scotian Shelf (1982–88)	0.66	0.03	0.87	0.02	0.76	0.03	Year	7
Scotian Shelf (1995–2004)	0.66	0.02	0.84	0.03	0.79	0.01	Year	10
Gulf of Maine	0.65	0.05	0.84	0.05	0.78	0.04	Year	21

ratios of stage lengths are, on the average, invariant. The regression lines in Figure 6 and ratios in Table 5 represent average stage lengths and growth rates over a wide range, bounded by the "cigar" shapes. Provided that there are enough samples to represent this average adequately, it should always converge to the same number.

These results suggest that it is the variance in growth rates and size of life history stages, not the invariance in the ratios between stages, which is most remarkable and informative about the biology of *P. borealis*. Although the ultimate mechanisms behind the evolution of a protandric life history strategy is beyond the scope of this paper, the present results suggest that this plasticity in growth rates and sizes of life history stages should be an important consideration in theoretical treatments, at least for *P. borealis*.

#### A Conceptual Growth Model for *P. borealis*

The two most remarkable and informative aspects of the stage structure of *P. borealis* are the observations that stage lengths are strongly related to each other within samples and that environmental, temporal and spatial factors influence the lengths of all stages. If the relationship between  $L_{50}$  and  $L_{max}$  (i.e., the apparent invariance of the ratio  $L_{50}/L_{max}$ ) results largely from growth process as suggested by Charnov and Skúladóttir (2000), Koeller et al. (2003) and this paper, one would expect this relationship to manifest itself only after the application of appropriate time lags to summarized data averaged by area and year. The achievement of maximum size in *P. borealis* follows sex change by at least two years, because there are at least two, and probably more, female age classes. Why is a relationship between life history stages separated in time, purportedly because of growth, so evident in individual samples where spatial, not temporal relationships should be most prominent? One obvious answer is that the observed within-sample relationships between stages do not result from growth but rather from schooling or migratory behavior that brings similar sizes together at the same locations. Ontogenetic migrations into deeper water have been inferred from the common observation that shrimp sizes (including average stage sizes) often increase with depth (reviewed in Shumway et al. 1985). Because larger shrimp are expected to migrate faster this mechanism would tend to segregate size groups along depth gradients. Another possible mechanism is vertical migration and associated horizontal displacement. Different sizes of shrimp tend to have different vertical migration ranges (Shumway et al. 1985), consequently a size group migrating vertically into layers with faster horizontal current speeds will be displaced farther than other groups. Over time this would have a "winnowing" effect, bringing shrimp of similar size to the same areas. However, this does not explain the temporal changes observed within areas, where length differences between years, or groups of years, tend to affect all stages (e.g., Scotian Shelf, Fig. 3B). In addition, it seems unlikely that such migrations or displacements, which must be quite variable between areas and times, would result in such invariant relationships between stages.

Assuming that the observed within-sample relationships between stages result from growth processes, these relationships could have come about only if growth rates of all stages (and ages) were highly sensitive and responsive to changes in local conditions, especially food availability and temperature. Shrimp of all sizes consume phytoplankton, detritus (i.e., senescent phytoplankton blooms), or small herbivorous zooplankton, therefore growth of all stages is strongly influenced by a common factor—food availability, as determined by primary production and shrimp den-

sity (Koeller et al. 2006, Fuentes-Yaco et al. 2006). In addition, shrimp of all stages at any location and time are subject to the same temperatures, arguably the second most-important modifier of growth rates (after food availability) in poikilotherms. Consequently, the growth rate of shrimp of all sizes, stages and ages present at any location will respond simultaneously to changes in the physico-biological conditions in the area, especially food availability and temperature. Moreover, in a situation where P/B ratios and natural mortality are high (M of 0.75 has been used in stock assessments), temporal changes in growth should become evident through a simultaneous change in all mean stage sizes almost immediately (i.e., in the same year). Significant correlations between environmental variables and the size (growth) of the later life history stages are therefore expected without time lags, as found by Fuentes-Yaco et al. (2006).

The strong positive relationship between depth and all shrimp stages off Newfoundland supports the conclusion that the within-sample relationship between stages is caused by growth processes. In this area depth is strongly (positively) related to temperature and can be used as a proxy for temperature, albeit without temporal components. In addition, the abundance of the high organic content sediments and particulate organic carbon deposition, which form an important food source for shrimp, tends to increase with depth (Ramseier et al. 2000). Because temperature and food availability increase with depth, growth rates are expected to do the same. Similar site (depth) specific growth rates caused by differences in local conditions have been observed for scallops in the Bay of Fundy (Smith et al. 2001). For shrimp, the strong relationship between environmental, temporal and spatial factors other than depth and all shrimp stages is further support for the conclusion that the within-sample relationship between stages arises because these factors influence the growth rate of all stages simultaneously and are highly site-specific.

The growth rate of *P. borealis*, a poikilotherm, is strongly influenced by temperature and food availability, both of which vary widely spatially and temporally in its huge geographic range (~2400 nautical miles between latitudes 40°N and 80°N). Consequently, growth rates must vary widely, spatially and temporally. Most eggs are produced by only 2-yr classes (F1 and F2 females). If sex change were triggered by size alone, a decrease in growth rate caused by changes in environmental conditions could delay sex change by a year or more before this size would be achieved, leading to a large decrease in egg production during the delay period if other factors affecting egg production, such as population size and spawning frequency, remain constant. Changing sex after a fixed number of years ensures a stable annual egg production. However, because the number of eggs a female produces is directly related to its size (Schumway et al. 1985), changing sex at a fixed age will result in significant decreases in egg production if growth rates are very slow. Consequently, the highly variable growth rates caused by environmental influences forces a compromise between stability and quantity in egg production. How might a sex changer manage this balance and become as successful in terms of abundance and geographical range as *P. borealis*? Because a fixed age or size at sex change coupled with highly variable growth rates can carry major penalties for fecundity, the answer must lie in flexibility. This flexibility is achieved if the size at sex change is fundamentally (i.e., physiologically) linked to growth and metabolic rates, which in turn are linked to environmental changes in temperature and food availability. This appears to be the case for *P. borealis* (Koeller et al. 2003, Charnov and

Skúladóttir 2000). Given that stage structures result from growth processes, the strong relationship between all stages (including size at sex change) shown in this study further support this theory. Formally and simply stated, sex change occurs earlier in time when growth rates are faster and later in time when they are slower.

Because environmental conditions tend to be relatively stable within limited geographical boundaries (e.g., within SFAs or stocks), growth rates, stage lengths and size/age at sex change vary less within subareas and stocks than between them. Under stable environmental conditions within-stock growth rates are similar and sex change occurs at the same year and size. However, the number of male year classes does change occasionally within stocks, either between environmental regimes (Fig. 5B, Koeller et al. 2003), or when only part of a large year class changes sex, a situation which is known to occur with large year classes, which tend to have slower growth because of density-dependant factors (Koeller et al. 2005, M. Aschaan, pers. comm. U. Skúladóttir, pers. comm.). If growth rates decrease greatly because of decreasing temperatures and food availability, sex change will come later in time. However, sex change is constrained to a short "window of opportunity" within the annual physiological/reproductive cycle, consequently if slower growth rates delay sex change in time this window may be missed and sex change delayed by a year. The opposite occurs if growth rates increase because of increasing temperatures and food availability. In this model the year and size at sex change are not magic numbers but simply the age and size a shrimp happens to be at the time sex change occurs, as dictated by growth/metabolic rates, and the yearly life cycle. Similarly, the relationship between the size at sex change and the maximum size achieved (i.e.,  $L_{50}/L_{max}$ ) are "invariant" only because growth rates are largely environmentally driven and affect the sizes of all stages including males, transitionals, females and  $L_{max}$ .

#### Limitations and Potential of Stage Structure Analysis

As used above, stage structure analysis allows for only qualitative, or semiquantitative observations on the relative differences in growth characteristics between areas and time periods (e.g., growth rates are slower or faster, longevity is longer or shorter). More sophisticated modeling approaches, such as tuning model results to observed stage structures, may provide more quantitative results, however, the present approach can still be useful in comparative studies, e.g. the Newfoundland-Labrador shelf problem described above.

The application of the model used in this study to infer growth characteristics of other invertebrates is limited. Modeled longevity is solely caused by differences in the number of male year classes, the number of female year classes is constant at 2, and the mortality of females older than 2 y is total. Although not completely correct in detail, this appears to capture the main aspects of *P. borealis* life history, as evidenced by the similarity of simulations and field observations. However, it is specific to protandic hermaphrodites such as *Pandalid* shrimp. Still, the general approach of comparing or tuning model life history stage and age structures with known growth characteristics may be applicable to non-sex changing invertebrate species with distinct life history traits that are fairly short in duration.

Stage structure must also be influenced by factors other than growth, which may limit its usefulness for making inferences on growth in certain situations. This is illustrated by the large differ-

ences in stage structure between commercial and survey samples (Fig. 7). Because they cover the same stock (Newfoundland-Labrador), these differences may be because of factors other than growth, in particular sampling time (throughout the year for commercial, late fall for survey), sampling strategies (seeking concentrations of large shrimp for commercial versus randomly distributed throughout its range for surveys) and fishing gear (square mesh cod-ends and Nordmøre selectivity grids for commercial, small mesh cod-end liners for survey). Despite these different methodologies much of the difference in stage structure in Figure 7 can still be attributed to differences in growth, specifically to different growth rates in the locations sampled by these two methods. Commercial fishing operations concentrate in deep water where larger shrimp of all stages are found, presumably caused by faster growth, consequently all stages are larger and the stage structure is straighter for commercial data. Nevertheless, some of the differences in lengths, especially for smaller stages like  $L_{min}$ , are probably caused by differences in cod-end mesh sizes. The site-specific nature of stage lengths/growth rates and the large differences between locations within years implies that within-year spatial variability must be accounted for when making between-year comparisons, especially for survey samples. Another important modifier of stage structure other than growth is the passage of large year classes through the population. Shrimp recruitment is strongly influenced by environmental factors (Koeller 2000) and this tends to be exacerbated in highly exploited populations, like the Gulf of Maine stock. However, the passage of year classes through the stage structure is sequential, and should be distinguishable from a change in growth rate, which tends to be simultaneous. Comparisons between survey series may be complicated by large differences in survey times, for example, comparisons between the spring-summer surveys in the Gulf of Maine and Scotian Shelf versus the fall-winter Newfoundland-Labrador surveys must take into account growth and other processes between survey times that may have influenced stage structure. More work is required to clarify the effect of various processes on population stage structure, including, but not necessarily restricted to, gear selectivity, mortality and recruitment i.e., the influence of strong year classes and associated density-dependant effects on growth rate.

Changes in the abundance of *Pandalid* species have been used as indicators of ecosystem regime shift in the North Pacific (Anderson 2000) and the North Atlantic (Koeller 2000, Lilly et al. 2000). In both oceans changes in *Pandalid* abundance are associated with both climatological change and predator abundance. Shrimp are uniquely situated trophically and are potential indicators of changes in both "top down" and "bottom up" processes throughout the marine ecosystem. In terms of the latter, their growth is affected by primary production processes, because they consume phytoplankton and small herbivorous zooplankton (Fuentes-Yaco et al. 2006). Climatological changes that affect water temperatures will also affect shrimp growth. A change in the growth rate of a shrimp stock as determined by a simultaneous increase or decrease in the carapace lengths of all stages may be an early indicator of fundamental environmental and ecological changes that will eventually affect other ecosystem components. The results in this paper suggest that the great plasticity of shrimp growth rates in response to environmental changes is a property that can also be used to practical advantage in fisheries and ecosystem management (i.e., as an early indicator of ecological change in the lower trophic levels).

## CONCLUSIONS

1. Stage structure analysis is useful in spatial and temporal comparisons of growth rates for *P. borealis*. It is more informative about growth rates than an examination of single stages, such as the size at sex transition,  $L_{50}$ , or the maximum size,  $L_{max}$ , in isolation.
2. When longevities do not change, as evidenced by a constant shape of the stage structure in time, a simultaneous increase in the mean length of all stages is indicative of an increase in growth rates, and vice versa.
3. The passage of a strong year class through the population can have a significant effect on stage structure, however it is a sequential event, distinguishable from a change in growth rate, which tends to affect mean size of all stages simultaneously.
4. Growth rates of *P. borealis* are highly site-specific and sensitive to environmental influences, particularly temporal and spatial changes in food availability and temperature.
5. The time of sex change is directly related to growth and metabolic rates (i.e., sex change comes later when growth rates are slow and earlier when growth rates are faster). Consequently, the age and length at sex change is highly flexible, and only constrained by the timing of the annual

reproductive cycle. This avoids the negative impacts of a fixed age or length at sex change on egg production in a species whose growth rates are dependent on highly variable environmental factors, and whose fecundity is strongly related to size.

6. The "invariant" relationship between  $L_{50}$  and  $L_{max}$  is not unique to these stages and simply a consequence of the strong relationship between environmental factors and growth rates of all stages.
7. The stage structures of *P. borealis* populations could be early indicators of fundamental environmental and ecological changes in the ecosystem.

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## EFFECTS OF TIDAL RESTRICTIONS AND POTENTIAL BENEFITS OF TIDAL RESTORATION ON FECAL COLIFORM AND SHELLFISH-WATER QUALITY

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**ABSTRACT** The relationship between artificial tidal restrictions and shellfish-water quality was studied within otherwise sparsely developed estuaries on Cape Cod (Massachusetts, USA). The primary study site, the 600-ha diked Herring River (Wellfleet, MA) estuary, has had a long history of shellfish-water closures because of water-column fecal-coliform (FC) contamination despite the lack of human fecal sources. Ongoing efforts to restore tidal flow to the system have raised questions about the effects on microbiological water-quality on extensive beds of wild oysters (*Crassostrea virginica*) and cultured hard clams (*Mercenaria mercenaria*) in the river mouth. This study combines observations of current spatial and temporal (tidal) patterns of water-column contamination with recent hydrodynamic modeling to predict the effects of proposed tidal restoration on shellfish-water quality. Under presently tide-restricted conditions, high FC is restricted to about 1000 m on either side of the dike structure and only during low tide, preventing the harvest of extensive natural oyster beds; farther downstream, hard-clam aquaculture is marginally protected by relatively coliform-free, high-salinity Cape Cod Bay waters. Modeling of Herring River under tide-restored conditions showed that a predicted 13-fold increase in river intertidal volume, over existing tide-restricted conditions, would dilute measured FC to concentrations that are acceptable for shellfish-growing waters. Restored tidal flow would also reduce coliform survival time by increasing salinity, dissolved oxygen and pH, all presently depressed throughout the system because of the biogeochemical disturbance of diking and drainage. Results from Herring River, plus a preliminary survey of other diked Cape Cod estuaries, suggest a direct relationship between the degree of tidal restriction and surface-water FC, which should be studied further.

**KEY WORDS:** fecal coliform, diking, salt-marsh restoration, aquaculture

### INTRODUCTION

Efforts to restore native habitats in historically tide-restricted salt-marsh estuaries primarily focus on removing artificial barriers to tidal exchange. In the context of past diking, filling and drainage, it is generally agreed that re-establishment of the original hydrology, hydrography, and salinity distribution is fundamental to restoring an estuarine ecosystem (Niering & Warren 1980, Burdick et al. 1997, Roman et al. 1995). Although this is an ecologically reasonable approach for restoring dominant estuarine processes and biota, actual restoration management must also consider human-land-use changes that can affect the nature, and social acceptability, of system response.

A most obvious recent land-use change along the US coast is the rapid increase in human population and development. One outcome of the development increase has been an apparent decline in microbiological water quality, typically monitored using coliform indicator bacteria. In particular, fecal coliform (FC) contamination has forced the closure of shellfish beds throughout the US coasts. Although this pollution is usually attributed specifically to an increase in development-related sources (e.g., agriculture, impermeable-surface runoff and wastewater, Mallin et al. 2001), coastal development has also likely affected bacterial transport and dilution, through altered coastal hydrography including tide restrictions.

Tides were first restricted in New England coastal wetlands, predominantly for road and railway construction and mosquito control, in the late 19th and early 20th centuries. Tide restrictions reduce the tidal prism and thereby limit seawater flushing and dilution of constituents delivered to the estuary in discharging freshwater; this dilution is reduced both landward and seaward of the restricting structure. In this purely physical way, tide restrictions may effectively increase fecal coliform concentrations in

coastal waters. Increasing development, road runoff and bacterial loading would have exacerbated the problem.

In addition, disturbance to salt marsh biogeochemical cycling and resulting water-quality degradation caused by diking could contribute to FC survival and management problems in diked wetlands. The blockage of tides reduces salinity; tide-restricted marshes are poorly flushed, leading to high oxygen demand and low summertime dissolved oxygen (Portnoy 1991); and drainage causes acid sulfate soil formation and low water-column pH (Gosling & Baker 1980, Sammut et al. 1995, Portnoy 1999). Low salinity increases coliform survival times in the environment (Carlucci & Pramer 1960, Goyal et al. 1977, Morinigo et al. 1990, Coelho et al. 1999, Lipp et al. 2001, Gabutti et al. 2000, Mallin et al. 2000, Bordalo et al. 2002).

Conversely, tidal restoration would reduce fecal bacteria concentrations, regardless of their source, first through simple dilution because of a much-increased tidal prism. In addition, increased salinity would reduce the survival time of enteric bacteria in the environment, as studies elsewhere have consistently demonstrated (Mallin et al. 1999, Coelho et al. 1999, Gabutti et al. 2000, Mallin et al. 2000, Lipp et al. 2001). Finally, laboratory and field experiments have shown that tidal restoration can quickly restore water-column pH (Portnoy & Giblin 1997a, Easton & Marshall 2000), further depressing coliform survival.

Chronically high fecal coliform and shellfish-waters closures just seaward of a large Cape Cod estuary planned for tidal restoration prompted this study. Extensive beds of eastern oyster (*Crassostrea virginica*, Gmelin 1791) up to 2 km seaward of the diked 600-ha Herring River estuary (Wellfleet, MA, USA) have been closed, at least seasonally, because of fecal coliform contamination since state-wide surveillance increased about 1983. With the largest hard clam (*Mercenaria mercenaria*, Linnaeus 1758) aquaculture beds in Massachusetts just seaward of the zone of chronic closures, there was public concern that tidal restoration could extend the FC problem to close a highly productive fishery. Coastal

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wetlands can be important sources of public-health indicator bacteria, reportedly because of the abundance of organic matter, nutrients and high water-column turbidity promoting bacteria survival (Jenson et al. 1980). Enterococci produced in a California tidal marsh affected surf-zone water quality under specific near-shore flow conditions (Grant et al. 2001).

The Herring River estuary is largely undeveloped, with very few cesspools or septic systems near the river and few road crossings, because of incorporation of 80% of the floodplain into Cape Cod National Seashore in 1961. Therefore, we began the study with the assumption that wildlife within the river basin was probably the major ultimate source of FC in surface water. Waterfowl and other birds can be abundant in or near the river and are probably a major ultimate source of coliform bacteria; these microbes can reportedly survive and perhaps grow in sediment (Gerba & McLeod 1976, Struck 1988). If this is the case for Herring River, bacteria could be resuspended with surficial sediment during periods of high freshwater discharge and/or tidal flow, to constitute an important proximate source to the receiving water column (Mallin et al. 1999, Grant et al. 2001). Because this source has not, given land-use history, recently changed, nor is it likely to substantially change with tidal restoration, we focused instead on the water quality and hydrodynamic factors that would affect coliform survival and transport under both existing and tide-restored conditions. The general goal was to use existing conditions together with recent hydrodynamic modeling results (Spaulding & Grilli 2001, 2005) to predict the effects of tide restoration on bacteria concentrations in downstream shellfish-growing waters.

Specific objectives were: (1) to describe where and when public-health bacteria contaminate presently restricted estuarine surface waters and (2) to predict how restored tidal exchange would affect shellfish-water quality.

## STUDY AREA

### Herring River

The 600-ha Herring River estuarine complex (42°N, 70°W), occupying a glacial outwash valley on the eastern shore of Cape Cod Bay (Fig. 1), is the largest diked wetland system on Cape Cod (Portnoy 1991). Tidal flow to most of the original salt marsh was eliminated by inlet closures in the eighteenth and nineteenth centuries, and by a dike built across the mouth of the main stream in 1909. Stream channelization and ditch drainage began before dike construction but intensified immediately thereafter; channel and ditch maintenance continued until 1984. The modern dike structure includes three 4-m<sup>2</sup> rectangular sluiceways providing a large opening for freshwater discharge (ranging 0.2–0.5 m<sup>3</sup> s<sup>-1</sup>) at low tide. During high tide, hydraulic pressure closes top-hinged doors (clapper valves) on two of the sluiceways, preventing seawater flow upstream. The third sluiceway is partially open (cross-sectional area about 1 m<sup>2</sup>) allowing some seawater entry; however, seawater flow up the main channel and onto the diked marsh surface is limited to 1,000 m upstream. The dike reduces tidal range from about 2.1 m seaward, to 0.5 m landward of the structure. At high tide in the river, saltwater extends only to Station 3; at low tide, salinity at the dike is about 15 ppt, decreasing to 0–3 ppt at Station 4 (Fig. 1). Because of the previously mentioned lack of tidal flushing and biogeochemical disturbance, the river above the reach of seawater suffers from low pH and low dissolved oxygen (Soukup & Portnoy 1986, Portnoy 1991). Approximately 15 ha of natural

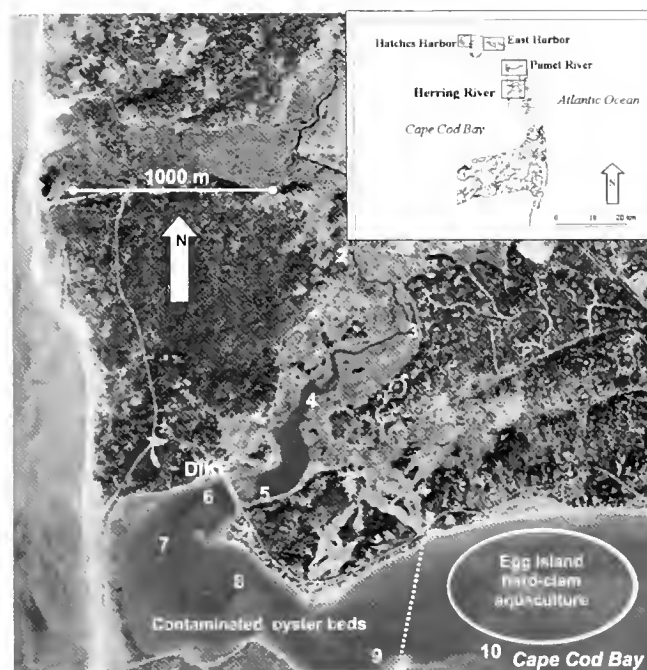


Figure 1. Herring River study area, water-quality sampling stations and location of hard-clam aquaculture and oyster beds. Inset: location of Herring River and other study sites on outer Cape Cod.

oyster beds and 20 ha of hard-clam aquaculture (Egg Island) at the river mouth, and seaward of the dike, are indicated on Figure 1.

Historic photographs taken just before the 1908 diking show an apparently continuous stand of salt marsh grasses along the shore of the Herring River. Core analyses by R. A. Orson (Roman 1987) documented the presence of smooth cord grass (*S. alterniflora*, Loisel) on the floodplain presently vegetated with velvet grass (*Holcus lanatus*, Linnaeus), meadowsweet (*Spiraea latifolia*, Ahles) and hardhack (*S. tomentosa*, Linnaeus) goldenrod (*Solidago* spp.) and black cherry (*Prunus serotina*, Ehrhart). Because of peat drainage, pore-space collapse and accelerated decomposition (Portnoy & Giblin 1997b), the marsh surface elevation is 0.95–1.01 m NGVD (National Geodetic Vertical Datum), or about 90 cm below modern unaltered *Spartina* marsh seaward of the Herring River dike.

Regarding sources of FC, impermeable surfaces comprise only two paved secondary roads crossing the floodplain, and there is only one domestic wastewater disposal system within 20 m of surface waters. Waterbirds, primarily waterfowl (Anseriformes), are seasonally abundant and probably a principal source of coliform in the river system (Valiela et al. 1991).

### Other Sites

The relationship between the degree of tidal restriction and low-tide FC concentrations was studied at several other Cape Cod estuaries in summer 2005. Pamet River (Truro, MA) has been completely tide restricted since about 1867, with no seawater passing through a dike that cuts off all tidal flow from the upper 64 ha of this wetland. The diked wetland is vegetated with freshwater-wetland shrubs, narrow-leaved cat-tail *Typha angustifolia* (Linnaeus) and common reed (*Phragmites australis*, Trinius).

East Harbor (Truro, MA) is a 145-ha back-barrier lagoon and 150-ha emergent wetland, also isolated by diking from the marine



environment since about 1868. Salinities have increased greatly in the East Harbor lagoon since 2002, from 4–20 ppt, when clapper valves in its drainage culvert were opened to improve flushing; however, tidal range remains <0.5 m in creeks immediately above the culvert, and 0 in the lagoon proper because of the small diameter of the culvert relative to lagoon volume. The East Harbor emergent marsh is vegetated with freshwater shrubs, *Typha angustifolia* and *Phragmites australis*.

Hatches Harbor (Provincetown, MA) is a 170-ha back-barrier salt marsh bisected by a dike in 1930; large culverts were installed in this dike and gradually opened beginning in 1999 so that to date (2005) nearly all restriction on tide height and salinity distribution has been removed. As a result of increased salinity, salt marsh herbs are replacing an extensive stand of *Phragmites*.

Like Herring River, these other coastal floodplains are within Cape Cod National Seashore, are consequently sparsely developed and have few sources of anthropogenic coliform pollution.

## METHODS

At Herring River, water and sediment sampling focused on nine sites spaced roughly 0.5–1.0 km apart along the main stem from above the reach of seawater (Station 2) to the mouth of the river at Egg Island (Station 10), a sandbar in Wellfleet Harbor used intensively for bivalve aquaculture (Fig. 1). Other estuaries were sampled within 50 m above and below road crossings that restricted tidal flow. Most sampling effort was directed toward FC bacteria because this group is the water-quality standard for shellfish-growing waters per the US Food and Drug Administration's National Shellfish Sanitation Program. Field-work, including periodic surface-water sampling and sediment collections, was conducted from May through October of 2005. Following preliminary surveys of FC throughout the tidal cycle, sampling focused on low tide to characterize worst-case conditions.

### Sediment

Sediment was collected by Ponar dredge on two dates at water-quality stations in Herring River. Dredged sediment was dropped upright into a basin and the top 2 cm transferred to a sterile cup using a flame-sterilized spoon. To control for expectedly high spatial variability, three randomly located grabs were collected at each sampling station and combined together in equal volumes.

In the laboratory, these composited samples were thoroughly mixed to homogenize. 20 g of wet sediment were removed aseptically, mixed with 200 mL of phosphate buffer, shaken for 45 min, and allowed to stand for 30 min. *E. coli* MPN (most probable number) in 100 mL of the supernate was determined using the Colilert-18 system (IDEXX Laboratories, Westbrook, Maine); incubation temperature was 35°C. Remaining sediment was dried at 105°C, dry-sieved for particle size distribution and combusted at 550°C, for 2 h to determine organic content. Bacteria concentrations were computed per unit of dry sediment weight.

### Water

Surface-water samples were collected aseptically at about 10-cm depth, transported to the laboratory at 4°C and cultured for FC using the membrane filtration method at 44.5°C on M-FC agar with rosolic acid (APHA 1998) within six hours of collection. All water samples were collected and cultured for FC in duplicate; data are presented as means  $\pm$  1 SD. On two dates (August 4 and 11, 2000), samples from all Herring River stations, plus Hatches

Harbor, East Harbor and Pamet River study sites were also tested for *E. coli* using the Colilert-18 system; as for sediment (above) incubation temperature was 35°C; results are presented as MPN (most probable number) per 100 mL. The Colilert system has been shown to perform as well or better than traditional membrane-filtration methodology for these bacterial indicators (Yakub et al. 2002).

Note that for Herring River sediment, *E. coli* was cultured, as opposed to FC in water samples; this was simply because an FC method was not available for sediment using the IDEXX system. We therefore do not directly compare densities of FC in water and *E. coli* in sediment, but rather compare their respective spatial patterns along the estuarine salinity gradient. *E. coli* is of course a major component of fecal coliform.

Salinity was measured by refractometer. Turbidity was measured with a Hach 2100P turbidimeter, calibrated with primary formazin standards.

## RESULTS

### Sediment

Highest *E. coli* concentrations were found in sediment at stations 200–1,400 m above the dike (Fig. 2). Because of the tide restriction, this is a reach of limited tidal range and flood-tide velocity; therefore, fine particles tend to deposit directly into the channel. In comparison, concentrations above this river reach were relatively low on both sampling dates. Immediately seaward of the dike, sediment bacteria were moderately high in July but low in August. From about 500 m of the dike structure to the channel south of Egg Island, *E. coli* was low to undetectable on both sampling dates. Bacteria density was related to sediment physical properties as measured at these sampling locations (Table 1): *E. coli* appeared highest in relatively fine-grained sediments with low bulk density and high water content.

### Surface Water

A preliminary survey in May 2005 of surface-water FC at 30 min intervals from midebb to midflood tide just seaward of the dike structure (Station 6, Fig. 1), showed that highest concentrations occurred at lowest salinities coinciding with low tide in the diked river (Fig. 3). In addition, a survey of all nine river stations at both low and high tides on the same date in October (Fig. 4) showed a similar relationship between tidal stage and FC: essentially, FC varied inversely with salinity at stations 5 through 10 from just above to 2,000 m seaward of the dike; FC at Stations 2–4 in the diked river proper was similar at both high and low tide. Given these observations, to characterize worst-case conditions most other water sampling was conducted at low tide.

Repeated low-tide sampling throughout the summer months corroborated the relationship between FC and position along the river main stem (Fig. 5): FC was low to moderate at Stations 2 and 3, high at Stations 4 through 8, about 1,000 m above and below the dike, respectively and very low at Stations 9 and 10. On September 18, Tropical Storm Ophelia delivered one of only two substantial (>1 cm) rain events during the summer to the study area. During the resulting freshet, low-tide FC increased 2- to 4-fold at Stations 2 through 8, and about 80-fold at Stations 9 and 10; however, the same relationship between FC and river reach was observed as during drier weather, albeit with much higher values, indicative of increased FC discharge from upstream wetlands (Jenson et al.

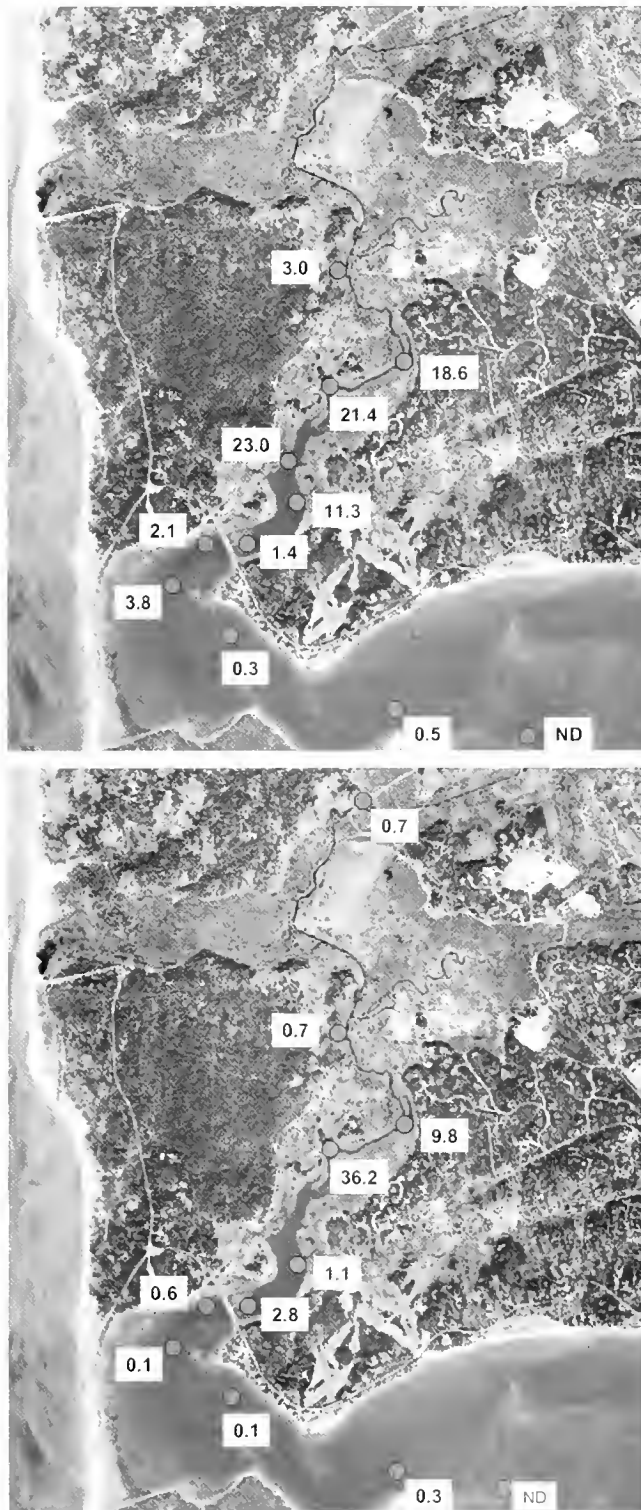


Figure 2. *E. coli* concentrations (colony forming units per g DW) in Herring River sediment on July 20 (top) and August 16 (bottom) 2005.

1980). Concentrations returned to background dry-weather conditions 5 days after the September 18 rain event.

Although highest low-tide, surface-water concentrations of FC consistently occurred at and just seaward of the river reach with highest sediment stocks of *E. coli* (Fig. 2), FC did not vary with

TABLE 1.

Correlation coefficients (Pearson's *r*) between *E. coli* concentrations and sediment physical properties at Herring River stations in summer 2005; *n* = 22. Significance levels are given in parentheses.

Collection Date	Median Grain Size	Water (%)	Organic Matter (% DW)	Bulk Density (g cc <sup>-1</sup> )
20 Jul	-0.638 (0.03)	0.610 (0.05)	0.496 (0.12)	-0.650 (0.03)
16 Aug	-0.514 (0.11)	0.430 (0.18)	0.286 (0.39)	-0.445 (0.17)

turbidity as from suspended sediment ( $R^2 = 0.08$ ,  $P < 0.05$ ). A plot of all turbidity and FC observations versus salinity showed a complex relationship (Fig. 6), with high turbidity at both freshwater and marine end members, and moderate and low FC respectively; lowest turbidity corresponded with highest fecal coliform concentrations at low-tide salinities of 10–25 ppt, typically within 1,000 m above and below the dike.

#### Physical Effects of Tidal Restoration

Hydrodynamic modeling, conducted to assess physical alternatives for tide restoration (Spaulding & Grilli 2001, 2005), showed that tidal range could increase from 0.5–2.0 m, and intertidal volume from about 73,000–980,000 m<sup>3</sup>. Assuming most of the water that exited the river during the ebb did not return during subsequent flood tides, as evidenced by consistently high flood-tide salinity, the increase in intertidal volume amounts to a 13.4-fold increase in the dilution of river water. Given this dilution factor, and assuming that bacterial loading does not increase, restored tidal flushing should reduce coliform bacteria to concentrations below the standard for shellfish waters (Fig. 7), except for low-tide periods 2–4 days after heavy rain.

#### Other Study Sites

Although seasonal use by waterfowl, larids and roosting passerines may be one factor contributing to high FC above the Herring River dike structure (Moles 2005), another appears to be the effect of tide restriction itself on water and sediment quality. This is supported by less intensive sampling in the other large diked salt marshes on Cape Cod. We noted on two sampling dates in mid-summer that low-tide FC and *E. coli* in the water column at Pamet River and both East and Hatches Harbors related directly to the degree of tidal restriction, indicated by depressed low-tide salinity (Fig. 8).

## DISCUSSION

#### Current Hydrographic Conditions

Chronically high surface-water FC occurred in the principal zone of river- and bay-water mixing, where low-tide salinities ranged from 10–25 ppt; this zone extended about 1,000 m on either side of the dike tidal restriction. Sediment in the upstream reaches of this zone (e.g., Stations 3 and 4, Fig. 1) had high *E. coli* and may have been the proximate source. Abundance of *E. coli* in Herring River sediment corresponded with an open-water habitat above the dike that was highly attractive to waterfowl and larids (Portnoy et

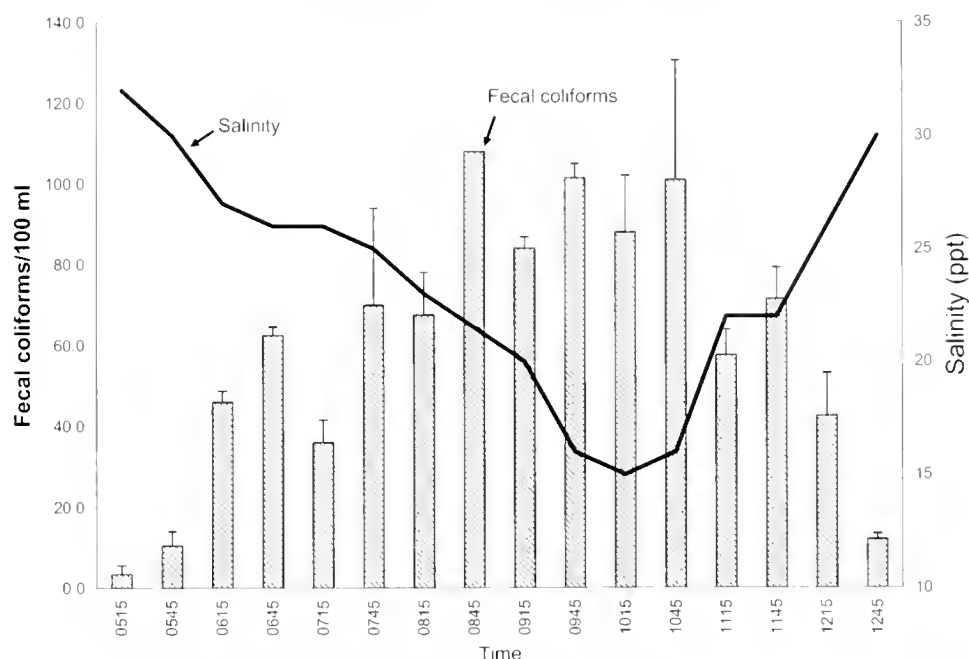


Figure 3. FC concentrations ( $\pm 1$  SD) and salinity in Herring River surface water from midebb to midflood tide, May 26, 2005.

al. 1987) and roosting passerines; it is likely that these animals seed the river sediment with coliform bacteria in their droppings. There is increasing evidence that fecal coliform can survive and even grow in the environment, especially in sediment (Gerba & McLeod 1976, LaLiberte & Grimes 1982, Struck 1988, Davies et al. 1995, Grant et al. 2001). Their survival time in sediment would be extended by low salinity (Mallin et al. 2000, Lipp et al. 2001, Anderson et al. 2005), low pH (Carlucci & Pramer 1960), and high sediment organic content (Gerba & McLeod 1976, Matson et al. 1978, LaLiberte & Grimes 1982)—the very conditions that prevailed in Herring River because of the restriction of tides and ditch drainage (Portnoy 1999).

FC concentrations in river surface water were clearly dependent on tidal stage and the amount of fresh water from the high-FC zone just above the dike relative to the high-salinity, and comparatively low-FC, Cape Cod Bay end member (Fig. 3, 4). Low-tide surveys consistently showed that high FC, exceeding the USDA shellfish-waters standard of 14 CFU (colony-forming units) per 100 mL, was the norm for all river stations; in contrast, dry-weather FC was always low at the most seaward Stations 9 and 10 (Fig. 5), demonstrating the predominance of Cape Cod Bay water in the salt and water budget of Wellfleet Harbor. It should be noted, however, that the protection afforded Wellfleet's Egg Island aquaculture industry by clean Cape Cod Bay water was marginal.

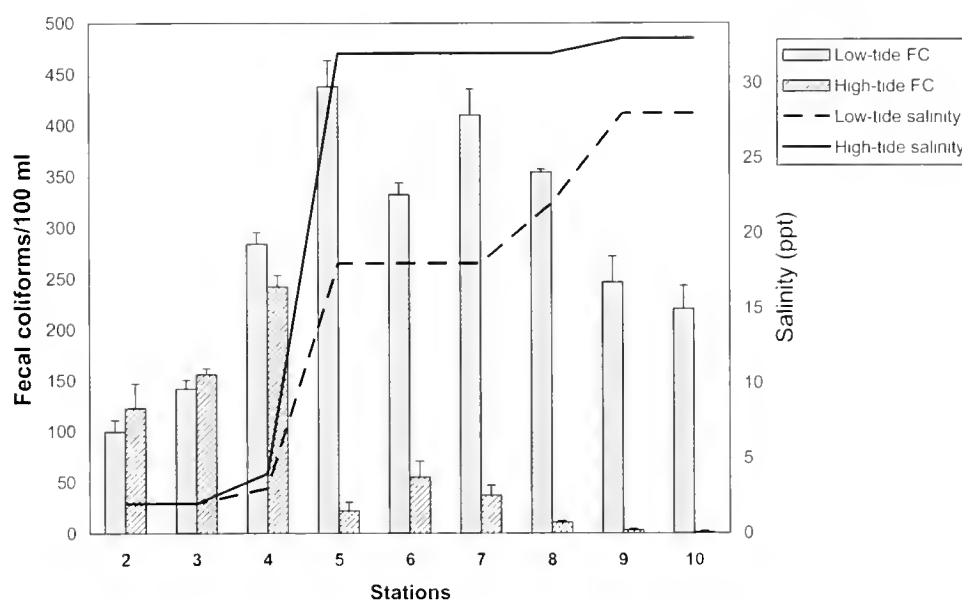


Figure 4. Low- and high-tide salinity and fecal coliform ( $\pm 1$  SD) in Herring River, October 20, 2005.

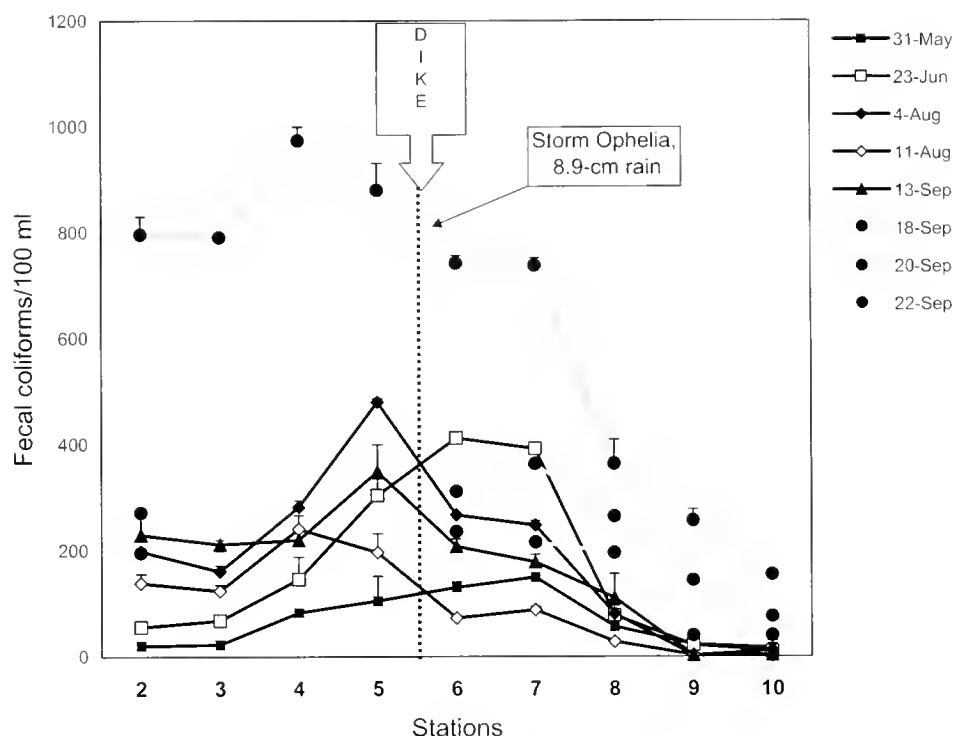


Figure 5. Low-tide FC ( $\pm 1$  SD) at Herring River stations throughout the 2005 summer including days one (Sep 18), three (Sep 20) and five (Sep 22) after a 8.9-cm rain event.

with observed violations of the water-quality standard during wet weather (Fig. 5). Meanwhile, under current tide-restricted conditions, the extensive oyster beds in the river mouth between Stations 5 and 9 (Fig. 1) were chronically contaminated and closed to shellfish harvest.

Fecal coliforms are often associated with sediment (Faust et al.

1975, Valiela et al. 1991) and can be released into the water column by sediment disturbance (Rittenberg et al. 1958, Matson et al. 1978, Coelho et al. 1999, Mallin et al. 1999, Mallin et al. 2000). This can be a concern in tide-restoration projects if hydrodynamic changes trigger resuspension of FC-rich sediment into bathing or shellfish waters. However, FC has not always correlated well with

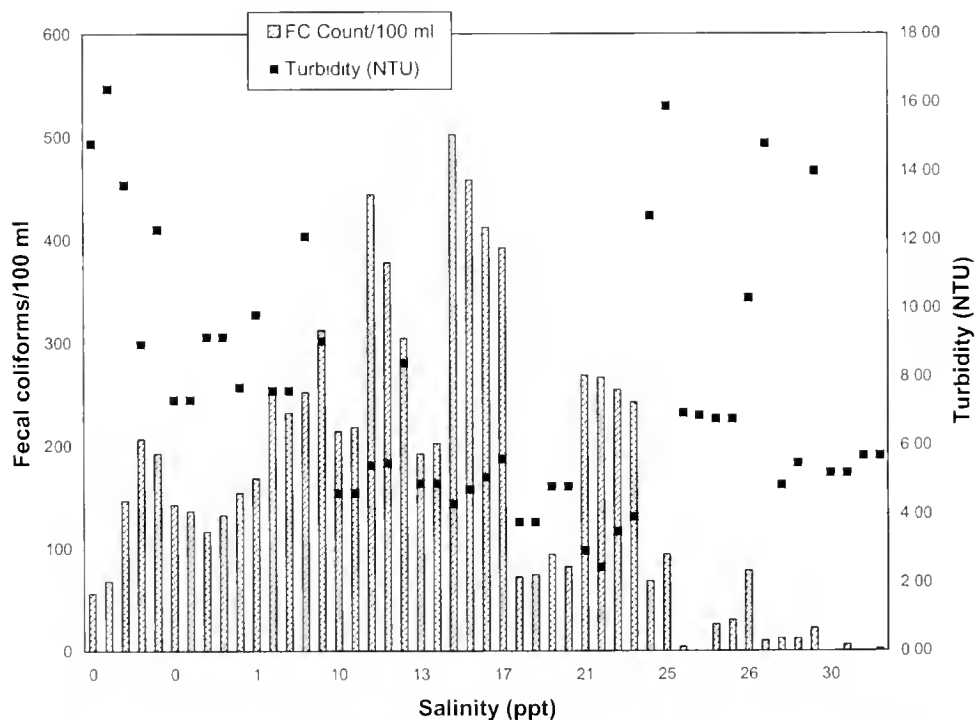


Figure 6. FC and turbidity across the Herring River salinity range, all summertime observations.

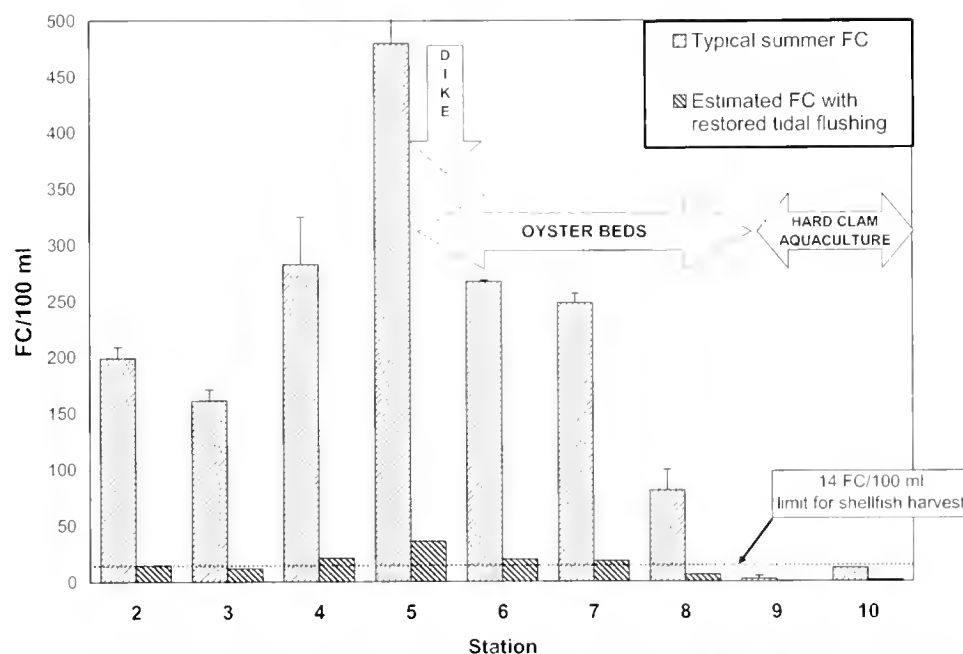


Figure 7. Estimated dilution effects on Herring River fecal coliform concentrations, relative to shellfish-waters standard, given model-predicted increase in intertidal volume and flushing with tidal restoration. Also shown are locations of oyster and hard clam beds relative to river sampling stations.

turbidity as a measure of sediment resuspension in studies elsewhere (Goyal et al. 1977, Jenson et al. 1980). Indeed, sediment resuspension did not appear to be an important mechanism affecting microbiological water quality under existing conditions in Herring River. Highest FC observed in the river channel corresponded with the lowest turbidities, and midrange salinities (10–25 ppt);

highest turbidities were at opposite ends of the salinity gradient (Fig. 6). The high turbidity at the river mouth (Stations 6–10) was apparently caused by the flocculation of dissolved organic matter and complexed Fe as salinity and ionic strength increased (Fig. 6), a process that is well described for rivers world-wide (Boyle et al. 1977); it was not the result of sediment resuspension. Thus, as

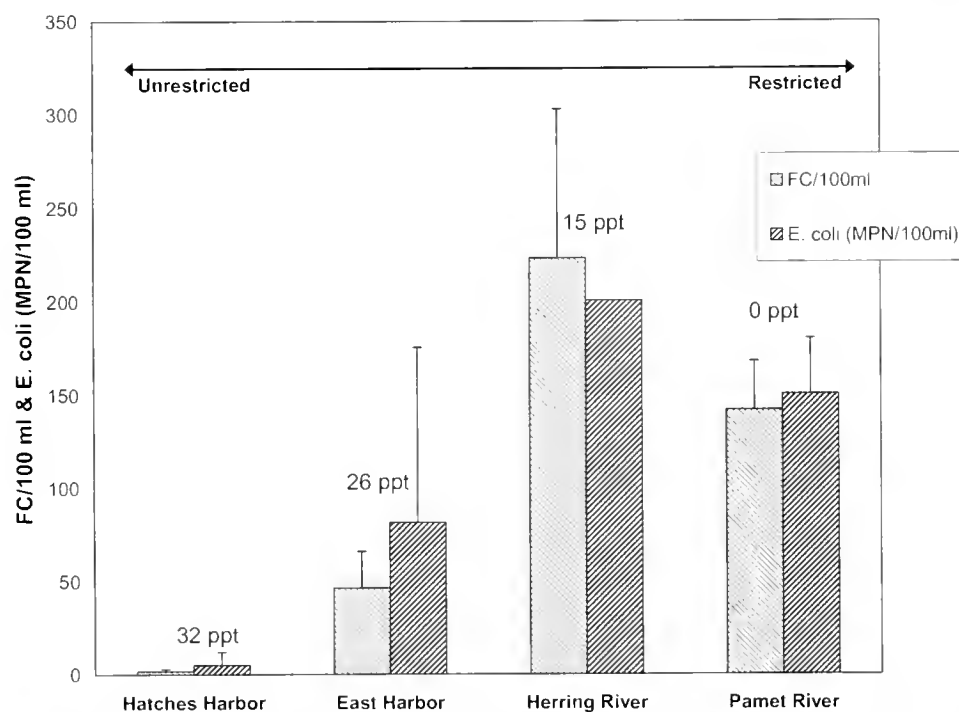


Figure 8. Typical summer fecal coliform and *E. coli* concentrations (mean  $\pm$  SD,  $n = 2$ ) in tide-restricted and restored Cape Cod estuaries at low tide. The degree of anthropogenic tidal restriction is indicated by low-tide salinity, indicated over bars; with no restriction, low-tide salinity would be about 30–32 ppt.

river water mixed with seawater at the river mouth during ebb tide, turbidity rose and FC declined. High FC at mid-salinity Stations 4–8 may have nevertheless been caused by tidal stirring in the river channel (Mallin et al. 1999) that was sufficient to resuspend a bacteria-rich surface film, but insufficient to lift enough sediment to increase measured turbidity significantly. Hydrodynamic modeling results indicate that channel velocities in a tide-restored river would be insufficient to resuspend sediment (see below).

#### Effects of Tidal Restoration

Besides dilution (Fig. 7), other factors associated with restored tidal flow would depress coliform concentrations even more in a tide-restored Herring River estuary. Restoration will cause a radical change in water chemistry (Portnoy 1999, Spaulding & Grilli 2001). As mentioned, the survival time of enteric bacteria is reduced by high pH (Carlucci & Pramer 1960) and high salinity (Goyal et al. 1977, Bordalo 2002). With tidal restoration, low-tide salinities just above and below the dike structure would increase from the present 20–25 ppt to 30–32 ppt (Spaulding & Grilli 2005), decreasing coliform survival. The resaturation of current acid-sulfate soils (Portnoy & Giblin 1997b) with seawater would rapidly (weeks to months) restore circum-neutral pH of marsh peat and receiving waters (Portnoy & Giblin 1997a). In addition, the physical alternative for restoration comprises a wide culvert, which will not only increase high tide heights but will also improve low-tide drainage (Spaulding & Grilli 2005), greatly improving tidal flushing with generally oxygen-saturated seawater; this should alleviate summertime oxygen stress in the diked estuary (Portnoy 1991). All of these water-quality factors should drive bacteria concentrations below that expected with simple tidal dilution. Meanwhile, although the hydrodynamic modeling indicated that channel velocities would not increase enough to resuspend potentially FC-rich sediment (Spaulding & Grilli 2005), somewhat increased flood-tidal flow velocity and much-increased tide heights would shift fine-sediment deposition from river channels to the wetland surface, where both dessication and light would depress FC survival (Bordalo et al. 2002). Finally, under existing tide-restricted conditions, Herring River sediments with highest FC content are flooded throughout the tidal cycle; tide restoration and better low-tide drainage would expose these sediments to solar UV radiation, further reducing viable bacteria (Fujioka et al. 1981, Sinton et al. 2002) on or near the sediment surface.

#### Other Study Sites

Although many other differences among Pamet River and Hatches and East Harbors could have affected enteric bacteria, results were consistent with relationships observed at Herring River between the degree of tidal restriction and microbiological water quality. All three tide-restricted sites, except recently tide-

restored Hatches Harbor, have established conditions that promote FC retention and survival: specifically depressed tidal flushing, water-column salinity, pH, aeration and increased deposition of fine sediments in main creek channels.

#### CONCLUSION

The source of fecal bacteria in outer Cape Cod's restricted, but otherwise mostly undeveloped, estuarine watersheds is probably wildlife. Because land use has changed little in many decades, and it is not likely to change significantly in terms of wildlife use of coastal wetlands, attempts to limit this coliform source are unlikely to succeed. Even if it is assumed that FC from wildlife is a good indicator of the presence of human pathogens (Griffin et al. 2001), there would probably be strong public opposition to discouraging wildlife activity. Also, waterfowl (at least) are most abundant during winter when shellfish harvest is limited (Valiela et al. 1991, Weiskel et al. 1996). As an alternate approach, this study suggests that the hydrologic restoration of tide-restricted salt marshes may help to improve shellfish-water quality among its many other social and ecological benefits (Roman et al. 1995, Burdick et al. 1997).

Regardless of the bacterial source, tide restriction has created water and sediment conditions behind dikes that favor coliform accumulation and survival. With limited flushing by relatively coliform-free Cape Cod Bay water, ebb tides carry bacteria-laden Herring River water to downstream shellfish beds, closing many hectares of oysters and threatening extensive hard-clam aquaculture. In addition, the dike's restriction on semidiurnal flooding with aerobic seawater has caused oxygen stress and low salinity; and effective peat drainage has led to high acidity—all of which extend bacteria survival times.

Given observed spatial and temporal (tidal) patterns of water-column fecal coliform abundance in the diked Herring River, the projected physical and water-chemical effects of tidal restoration should cause a substantial improvement in bacteriological water quality. Aside from increases in salinity, dissolved oxygen and pH that should reduce enteric bacteria survival, dilution alone, from radically increased intertidal volume, should reduce coliforms to concentrations that allow reopening of oyster beds closed for many decades. In addition, increased sediment exposure during predicted lower low tides would further reduce bacteria survival. Assuming no new major source of FC within the river system, increased dilution of river water would confer even more protection on hard-clam aquaculture beds from bacterial contamination.

The relationships among tidal restrictions, water quality and public health bacteria should be studied in other coastal ecosystems, particularly those experiencing shellfish-bed closures. These results suggest that hydrodynamic and hydrographic alterations may contribute substantially to coastal coliform contamination even without dense urbanization.

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## TAQMAN® MGB REAL-TIME PCR APPROACH TO QUANTIFICATION OF *PERKINSUS MARINUS* AND *PERKINSUS* SPP. IN OYSTERS

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**ABSTRACT** Several molecular diagnostic assays have been developed in an attempt to replace the traditional Ray's Fluid Thioglycollate Medium (RFTM) assay for detection and quantification of *Perkinsus marinus* in oysters. Real-time PCR technology is a state-of-the-art method currently used to diagnose disease intensity in vertebrates. We developed a simple (two-reagent) real-time PCR assay to quantify *P. marinus* (PMAR) and *Perkinsus* spp. (PERK) in oysters, using TaqMan® assays designed with Minor Groove Binder (MGB) probes on an Applied Biosystems 7500 Real-Time PCR System. Both PERK and PMAR assays demonstrate strong correlations ( $R^2 \geq 0.99$ ) between parasite cell density and real-time PCR threshold cycle ( $C_T$ ) with amplification efficiencies  $\geq 99\%$ . The PERK assay results in similar amplification plots for the three species tested (*P. marinus*, *P. olseni* and *P. chesapeaki*), whereas the PMAR assay detects only *P. marinus*. A strong correlation ( $R^2 > 0.90$ ) was found between infection level determined by the traditional RFTM method and quantification by real-time PCR, based on internal standards prepared from *P. marinus* spiked oyster tissue. The PCR assays also detected *Perkinsus* in oysters diagnosed as negatives using the traditional method, suggesting that the described assay may be more sensitive. These assays provide a nonsubjective, specific and accurate quantification of *P. marinus* in oyster tissues and thus could potentially replace the traditional method in some applications.

**KEY WORDS:** TaqMan®, real-time PCR, *Perkinsus*, oysters

### INTRODUCTION

The long and continuing decline of eastern oyster (*Crassostrea virginica*) fisheries has been partly attributed to the persistent and oftentimes catastrophic effects of the diseases MSX (caused by *Haplosporidium nelsoni*) and Perkinsosis (caused by *Perkinsus marinus*). The ability to develop and execute effective management strategies has necessitated the development of efficient tools by which the prevalence and intensity of infections can be evaluated in natural populations. Traditionally, *P. marinus* infections have been assessed using the Fluid Thioglycollate Medium (RFTM) assay (Ray 1966). This method involves the culture of the parasite prior to staining and visualization, and thus it necessitates that the parasite be viable at the time of processing. Several molecular assays have been developed that not only streamline the diagnostic process but also can be used to assess prevalence of *Perkinsus* spp. in archived or frozen tissues, which could expand our understanding of the patterns in disease occurrence and the environmental factors that may influence them (see review by Villalba et al. 2004). Primers that target either the nontranscribed spacer (NTS) or internal transcribed spacer (ITS) regions of the ribosomal RNA (rRNA) gene complex have been used in *P. marinus* specific PCR assays (Marsh et al. 1995, Robledo et al. 1998, Audemard et al. 2004). General primers have also been designed to detect virtually all *Perkinsus* species (Casas et al. 2002).

Real-time PCR technology is a state-of-the-art method used to quantify initial target levels of RNA and DNA and has gained wide use in clinical laboratory diagnostics (Kaltenboeck & Wang 2005). Whereas standard PCR methods analyze product formation during the plateau phase (or endpoint), real-time PCR quantification occurs during logarithmic amplification of the target, which can be more accurately related to initial target concentration. This difference is critical for the development of assays comparable to the

RFTM method, which provides not only presence/absence of the parasite but also infection intensity. The utility of real-time PCR approaches in quantifying *Perkinsus* spp. was demonstrated by Audemard et al. (2004), who reported real-time PCR methods for the detection and quantification of *Perkinsus* in environmental samples.

Here, we report the development of a simple, efficient real-time PCR assay to quantify *P. marinus* and *Perkinsus* spp. cell density in oyster tissues using TaqMan® Assays, designed with Minor Groove Binder (MGB) probes, detected on an Applied Biosystems 7500 Real-Time PCR System and analyzed with SDS software. This real-time PCR method overcomes a common problem in real-time PCR protocols, specifically the need for efficient amplification in order for the assay to be accurate in the quantification of the target. The TaqMan® Assays pair specific forward and reverse PCR primers to amplify a relatively short amplicon (<150 bp). The targeting of a small amplicon serves to one, increase amplification efficiency and two, reduce false negatives that may result from DNA rearrangement events that can occur within larger target regions, which is particularly important when targeting the NTS or ITS regions. The specificity of the real-time PCR measurement is increased with the TaqMan® internal probe prepared with a 5' fluorescent reporter dye, a 3' quencher and an MGB tail to allow increased stringency (Kutyavin et al. 2000). The fluorescence of unbound probe is quenched until the probe binds to the amplicon and the 5' exonuclease activity of *Taq* polymerase permanently releases the 5' fluorescent signal. If the probe is released without having been cleaved, as in the case of mismatches encountered during the exonuclease activity, the two ends will resume their close association and no signal will be delivered.

The increased specificity provided by the inclusion of TaqMan® probes allows discrimination of comparatively minor differences between sequences. The combination of these factors (short target amplicons, specific probes) results in a sensitive, specific and accurate determination of *P. marinus* and *Perkinsus* spp.

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cell density in oyster tissue that not only increases the efficiency of diagnosing *Perkinsus* spp. in oysters but also provides a new tool that may extend our understanding of this important oyster pathogen.

## MATERIALS AND METHODS

### In vitro Cultures

*P. marinus* (P-1) cultures were obtained from J. La Peyre (Louisiana State University) and maintained as previously described (Gauthier & Vasta 1995). Cultured *P. olseni* and *P. chesapeaki* were obtained from the American Type Tissue Culture Collection (ATCC 50984 and 50807, respectively) and were used to test assay specificity.

### TaqMan® Assay Design

The assays were designed and synthesized as Custom TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA) and the target sequences for the designs were submitted after having been formatted with their File Builder software.

Two target sequences were chosen for assay design (Fig. 1), both within the ITS region previously targeted by Casas et al. (2002), and referenced by Audemard et al. (2004). After aligning these regions with similar regions from other *Perkinsus* species (obtained from GenBank), one 231 bp region was identified that appears to be conserved across all species within this genus, and the *Perkinsus* spp. specific (PERK) assay was designed to anneal within this region. The second target sequence was a region that appears to be conserved in all *P. marinus* records but contains a higher frequency of substitutions relative to other species. The first *P. marinus* specific (PMAR) assay was designed to anneal within this region. *P. olseni* was identified as having the most similar sequence in the PMAR region and was chosen for testing the specificity of the PMAR assay despite the low probability of its occurrence in local oysters. Other species, such as *P. chesapeaki*, are considered more likely to coinfect oyster populations in North Carolina but exhibited greater differentiation relative to *P. marinus*.

Although the PERK assay efficiently reported all *Perkinsus* species tested (see results) and the first PMAR assay efficiently reported *P. marinus*, the initial PMAR assay exhibited cross-reactivity with *P. olseni* samples. This could be attributed to the fact that there was only a single-base mismatch in the probe bind-

ing site in *P. olseni* and only a single-base mismatch in each of the two primers. To enhance the specificity, two of the oligonucleotides in the PMAR assay were redesigned. The first was a slight shift of the reverse primer to incorporate one additional base mismatch, whereas retaining its melting temperature ( $T_M$ ) of about 60°C. The other modification was to reduce the  $T_M$  of the probe. Ideally the MGB probes are designed to have a  $T_M$  of 70°C (Applied Biosystems, Foster City, CA). However, to provide greater discrimination between a perfect match to *P. marinus* and the slight mismatch to *P. olseni*, the probe was designed with a  $T_M$  of about 66°C. The changes in the PMAR primers and probes did not increase the potential for cross reactivity with the other *Perkinsus* species. For example, when compared with the *P. chesapeaki* sequence, the new PMAR forward primer contained 4 mismatched base pairs, the reverse primer contained five mismatched base pairs, and the probe contained six mismatched base pairs—more than sufficient to avoid the generation of a false positive.

### Preparation of *P. marinus* Standards for Real-time PCR

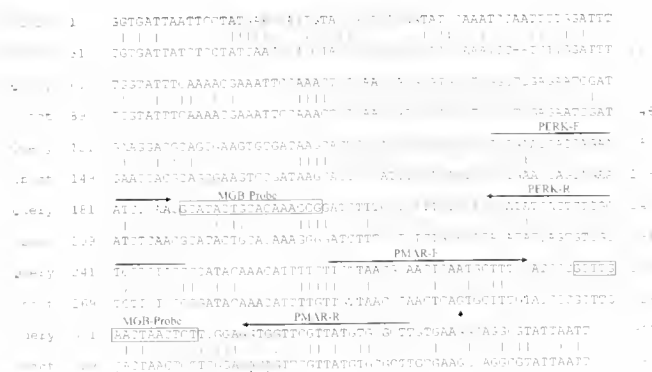
Cell density of *P. marinus* stock culture was determined by triturating cells through a 25-gauge needle and 5-mL syringe to break-up clusters prior to performing replicate hemocytometer counts. Replicate preweighed uninfected oyster mantle tissue samples (courtesy of Horn Point Laboratory, University of Maryland) were inoculated ("spiked") with 5, 50, 500, 5,000 or 50,000 *P. marinus* cells in a 10-μL volume of culture medium for the preparation of parasite cell density standards (parasites/g wet weight oyster tissue). These spiked tissue samples were immediately lysed and DNA extracted as described below.

### DNA Extractions

Oyster tissue samples were resuspended in 400 μL PureGene Cell Lysis Solution (D-5002) along with 2 μL of a 20-μg/mL stock solution of proteinase K and incubated at 55°C overnight or until all tissue was dissolved. Solutions were allowed to reach room temperature prior to the addition of 140-μL PureGene Protein Precipitation Solution (D-5003), vortexed vigorously for 20 sec and placed on ice for 5 min. Tubes were centrifuged at top speed on a microcentrifuge for 5 min and supernatant transferred to a clean labeled tube. The DNA was precipitated with the addition of 400-μL 100% isopropanol then gently inverted 50 times prior to centrifugation for 5 min. The DNA pellet was washed with 400-μL 70% ethanol and allowed to air dry prior to the addition of 35-μL double deionized water (ddH<sub>2</sub>O). DNA concentration (μg/μL = A260 nm × 0.05 × dilution factor) was determined on a Genesys 8 spectrophotometer and all samples were adjusted to 100 ng/μL. The 260-nm/280 nm absorbance ratio was used to determine the purity of DNA.

### TaqMan® Reagents

The PERK specific primer/probe mix containing 18 μM each of forward and reverse primers and 5-μM TaqMan® probe, which was obtained as a 20× stock solution (ABI 4331348, Applied Biosystems, Foster City, CA). The PMAR specific primer/probe mix was prepared manually by diluting each 100-μM primer (ABI 185312336–1,2) and 100-μM probe (ABI 185312336) to 18 μM and 5 μM, respectively for a 20× stock solution. The TaqMan® Universal PCR MasterMix (ABI 4324018) containing AmpliTaq Gold DNA Polymerase, AmpErase without UNG, dNTPs with dUTP, and buffer components optimized as a 2× solution.



**Figure 1.** Primer and probe design for PERK and PMAR real-time PCR assays within the ITS region of *Perkinsus* spp. rRNA. The top sequence is *P. marinus* and the bottom sequence is *P. olseni*. Arrowheads highlight mismatches between sequences.

### Real-time PCR

Reagents were added in the following proportions to each well of 96-well optical reaction plates (ABI 4306737): 12.5- $\mu$ L MasterMix, 1.25- $\mu$ L primer/probe, 9.25- $\mu$ L ddH<sub>2</sub>O, and 2- $\mu$ L of template (100  $\mu$ g/ $\mu$ L) for a 25- $\mu$ L reaction volume. Plates were sealed with ThermalSeal RT (Excel Scientific, Inc). Real-time PCR was conducted on an ABI 7500 Real-Time PCR System using the ABI 7500 System SDS Software using the default temperature program (95 °C/10 min; 40 cycles of 95 °C/15 sec followed by 60 °C/1 min) for a total run time of 1.5 h.

### Specificity and Sensitivity of PERK and PMAR Assays

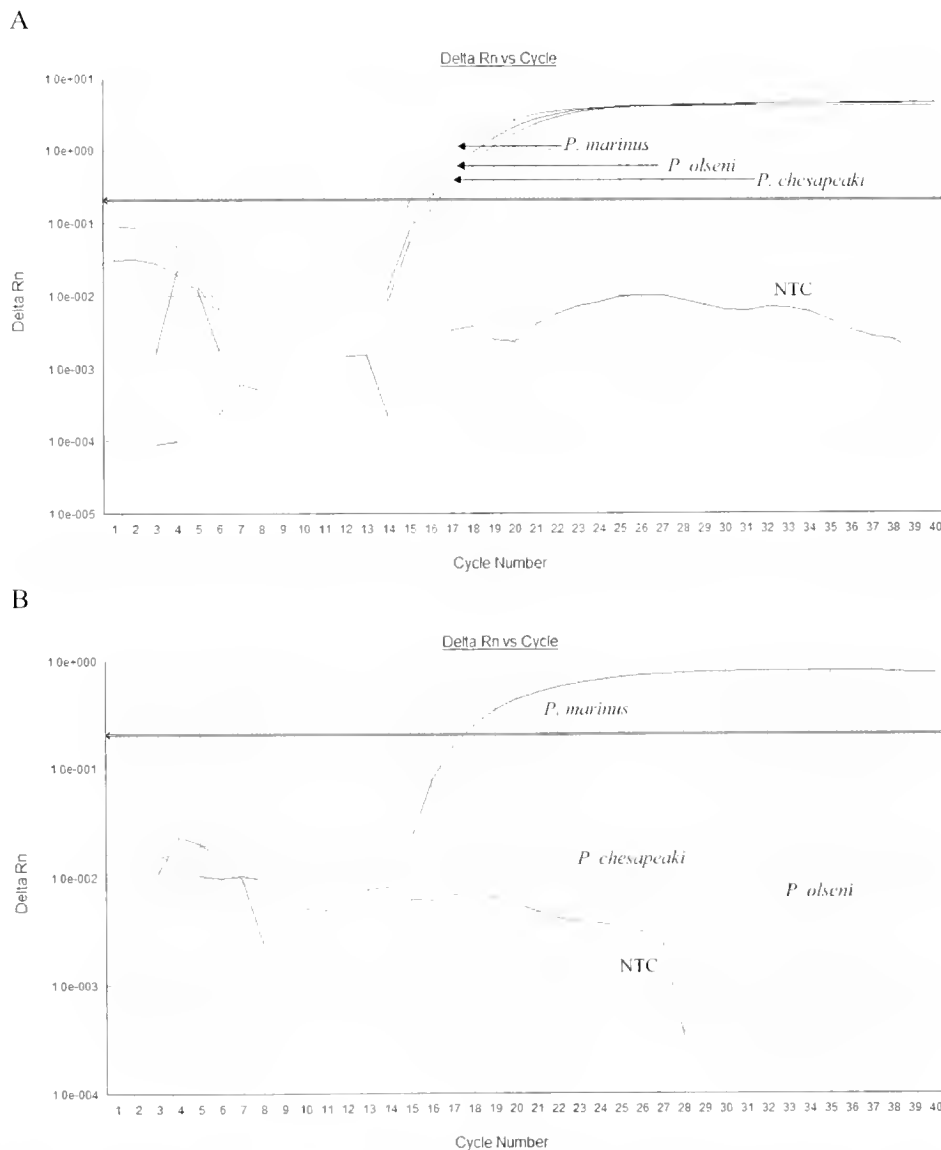
The specificity of PERK and PMAR primer/probe reagents was determined by conducting real-time PCR as described above on separate aliquots of 10-ng DNA from *P. marinus*, *P. olseni* and *P.*

*chESApeaki* and observing each of the amplification plots relative to nontemplate controls. The sensitivity of the two assays was determined separately on 10-fold serial dilutions of *P. marinus* target DNA (10 pg-0.001 fg) in the presence of 100 ng oyster DNA.

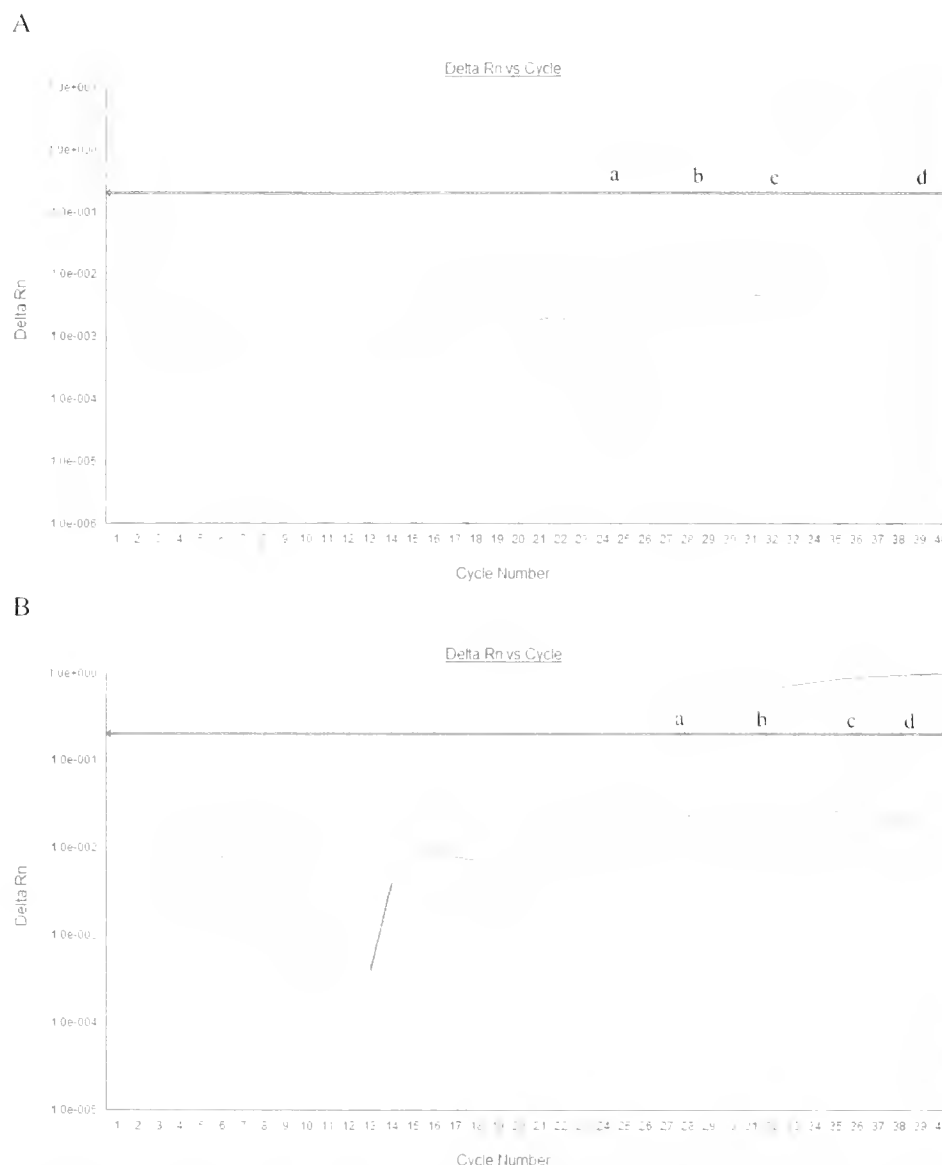
### Quantification of *Perkinsus* spp. in Naturally Infected Oysters

A comparison was made between *P. marinus* infection intensity determined by real-time PCR and the traditional RFTM assay using mantle tissue. Thirteen intertidal oysters were collected from Masonboro Sound at the UNCW Center for Marine Science (30 ppt, 24 °C ambient conditions). Two ~5mm<sup>2</sup> pieces of mantle tissue were removed and placed in either 10-mL RFTM medium with antibiotics for the traditional method (Ray 1966, Mackin 1962, Craig et al. 1989) or microcentrifuge tube for real-time PCR analysis.

Determination of parasite cell density in unknown samples was



**Figure 2.** Specificity of PERK (A) real-time PCR assay demonstrated by amplification of *P. marinus*, *P. olseni* and *P. chESApeaki* and PMAR (B) real-time PCR assay demonstrated by amplification of *P. marinus* but not *P. olseni* or *P. chESApeaki*. NTC = nontemplate control. Positive amplification is indicated when Delta Rn values exceed the threshold indicated by the horizontal line. Delta Rn represents the change in fluorescent signal produced from the cleaved TaqMan<sup>®</sup> probe, reporting successful binding of the probe to the target sequence.



**Figure 3.** Detection limit (10 fg) of PERK (A) and PMAR (B) real-time PCR assays demonstrated by amplification plots for a 10-fold serial dilution of *P. marinus* DNA in the presence of 100 ng of uninfected oyster DNA. a = 10 pg; b = 1 pg; c = 0.1 pg; d = 10 fg. Positive amplification is indicated when Delta Rn values exceed the threshold indicated by the horizontal line. Delta Rn represents the change in fluorescent signal produced from the cleaved TaqMan<sup>®</sup> probe, reporting successful binding of the probe to the target sequence.

accomplished by including three or more standards (described above) for each real-time PCR run. The ABI 7500 software automatically plots the relationship between cell density and threshold cycle ( $C_T$ ) and converts  $C_T$  values of unknowns to parasite cell density. The  $C_T$  value represents the number of cycles required for target amplification to reach the exponential phase and is therefore inversely related to initial target concentrations.

#### Statistical Analysis

Linear regression analysis was conducted to determine the relationship between parasite cell density and  $C_T$  value and between real-time PCR determination of infection intensity and semiquantitative estimates using the RFTM method.

## RESULTS

### Specificity and Sensitivity of PERK and PMAR Assays

The PERK assay resulted in similar amplification plots for *P. marinus*, *P. olseni* and *P. chesapeakei* DNA (Fig. 2A), whereas the PMAR assay resulted in amplification of *P. marinus* but not *P. chesapeakei* or *P. olseni* (Fig. 2B). The sensitivity of the PERK and PMAR assays were similar, detecting down to 10 fg *P. marinus* DNA in the presence of 100-ng oyster DNA (Fig. 3).

### Standard Curves for PERK and PMAR Assays

A strong correlation ( $R^2 \geq 0.99$ ;  $P < 0.0001$ ) was observed between *P. marinus* cell density in oyster tissue and  $C_T$  determined

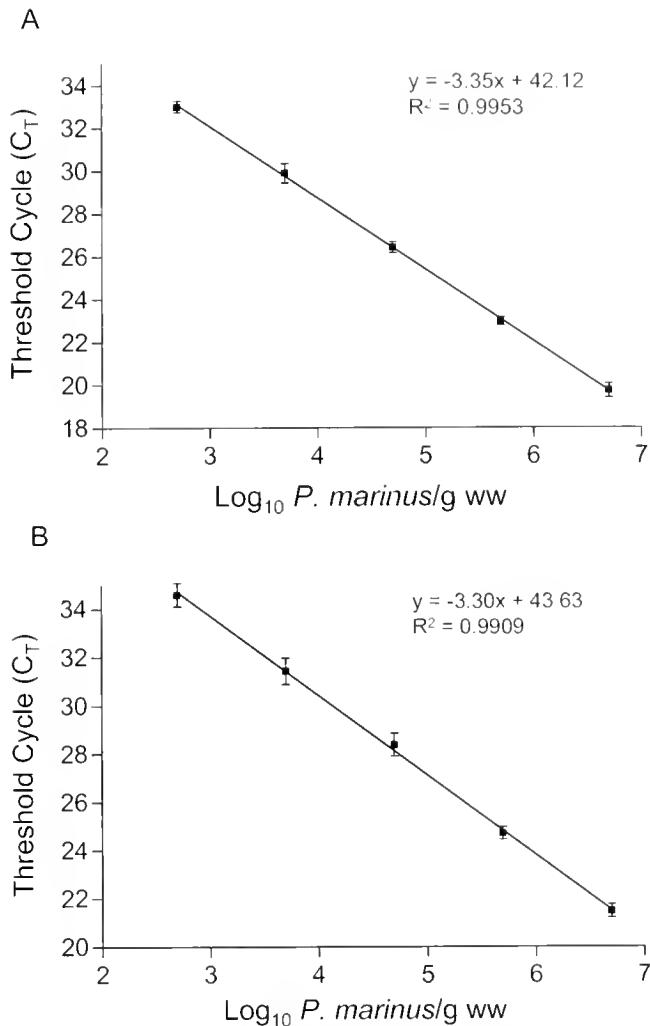


Figure 4. Standard curves representing relationship between parasite cell density of spiked oyster tissue ( $\log_{10}$  parasites/g wet weight oyster tissue) and threshold cycle ( $C_T$ ) determined for the PERK (A) and PMAR (B) real-time PCR assays. (Mean  $\pm$  SE;  $n = 2$ )

by PERK and PMAR real-time PCR assays (Fig. 4). Amplification efficiency ( $[10^{(-1/\text{slope})} - 1]$ ) for both assays was determined to be  $\geq 99\%$ . These parasite cell density standards were included in all PCR runs to determine infection intensity in field collected oyster tissue described later.

#### Quantification of *P. marinus* in Naturally Infected Oysters

A strong correlation ( $R^2 \geq 0.90$ ;  $P < 0.0001$ ) was found between infection intensity determined by the traditional RFTM mantle tissue assay and both PERK and PMAR real-time PCR assays based on internal standards prepared from *P. marinus* spiked oyster tissue (Fig. 5). Both PCR assays detected parasites in oysters (2 out of 13) diagnosed as uninfected by the traditional RFTM method. All 13 oysters tested positive by PCR, but only 11 tested positive by the RFTM method.

#### DISCUSSION

The results presented suggest that the TaqMan<sup>®</sup> MGB approach provides an efficient and accurate method for not only quantifying the prevalence and intensity of *P. marinus* infections

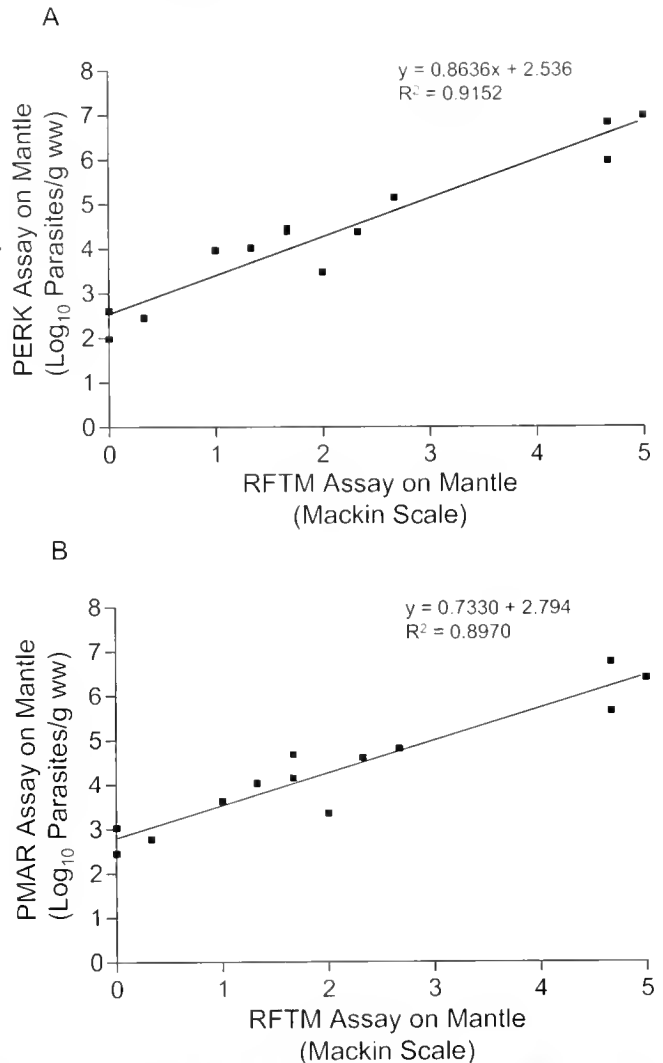


Figure 5. Relationship between the traditional RFTM assay for determining *Perkinsus* spp. infection intensity and PERK (A) and PMAR (B) real-time PCR assay determination of parasite cell density in mantle tissue.

but also a tool for investigating patterns of coinfection involving other species of *Perkinsus*. The combination of the genus-specific (PERK) that targets an 86 bp region and the species-specific (PMAR) assays that targets a 72 bp region can be used to evaluate the extent to which positive *Perkinsus* diagnosis could be attributed to species other than *P. marinus*. Although not all reported *Perkinsus* species were tested, the PERK assay recognized both *P. marinus* and *P. chesapeakei*, which share the lowest sequence identity within the target region, based on the data available in GenBank. The specificity of the PMAR assay is demonstrated by the lack of recognition for *P. olseni*, the species with the greatest sequence identity to *P. marinus* within the target region.

Our results demonstrate that the PCR assays are an accurate alternative to the traditional mantle smear RFTM method for the assessment of disease intensity. Parasite cell density standards, prepared by spiking oyster tissue samples of known wet weight with known numbers of cultured *P. marinus*, were used to determine parasite density in naturally infected oysters and these estimates correlate well with those obtained using the RFTM method

on the same oysters. Further, the linear relationship between the two methods allows us to convert the PCR data to familiar terms (Mackin Scale) if needed. Such agreement between methods allows for integration of data sets that rely on the different methods.

The reliance of the assay on the relatively easy to sample mantle tissue is also advantageous. Research has shown that although mantle tissue can be used fairly accurately to assess disease prevalence and intensity in oyster populations (Bushek et al. 1994, Oliver et al. 1998), the traditional RFTM method is known to result in false negative diagnoses at low infection levels (Choi et al. 1989). A major advantage of the PCR approach is the increased sensitivity with which mantle tissue samples can be analyzed, thus minimizing the likelihood of a false negative diagnosis. In this study, PERK and PMAR assays detected infections in oysters that were diagnosed as negative by the RFTM method.

Although the traditional RFTM method is approximately 3-fold less expensive, there are several advantages and particular applications of the real-time PCR assays: First, they can be used to distinguish between *P. marinus* and other *Perkinsus* spp. The ability to determine the extent to which infected oysters harbor more than a single species is of considerable interest. Nonspecific quantification could result in overestimation of *P. marinus* levels in areas where different species co-occur. Whereas the small numbers of natural oysters evaluated during this study exhibited similar infection intensities when tested with the generic and specific assays (suggesting an absence of multiple infections), a more substantial study would provide a more definitive answer to this question. Further, the ability to specifically attribute infections to *P. marinus* may facilitate the evaluation of other species to act as potential reservoirs.

A second advantage is that the PCR assays eliminate the subjectivity associated with estimation of infection intensity using a semiquantitative rating system. The potential for subjective error poses a problem when comparing year-to-year samples, particularly when there are multiple investigators involved. Third, the assays are extremely sensitive, detecting down to 10 fg of *P.*

*marinus* DNA in the presence of 100 ng oyster DNA. This sensitivity is likely to result in a more accurate assessment of disease prevalence and intensity. Fourth, the assays provide an accurate, quantitative measure of parasite cell density in oyster tissue samples. The linear relationship between known parasite cell density and  $C_T$  is used to quantify infection level in unknown tissue samples. Fifth, these assays can be accomplished in 1–2 days if needed as opposed to 5–7 days required for the RFTM assay. The assays are also relatively simple molecular protocols with only two reagents involved in setting up the PCR reaction. Lastly, the assays can make use of frozen samples or archived DNA, whereas the RFTM method requires that parasites be kept alive until processing. This attribute is allowing us to assess oyster disease patterns in North Carolina over the past several years from archived samples. Oyster tissue can also be sent through the mail in cell lysis solution without refrigeration for processing elsewhere.

The advantages associated with the adoption of real-time PCR strategies for the quantification of Perkinsosis in oysters may be offset in part by the greater expense associated with such techniques. Further refinement and automation of the assay will undoubtedly reduce the costs, and the advantages of the real-time assays will, in some cases, more than justify the additional costs. The linear relationship between the PCR assay determination of parasite cell density and the traditional semiquantitative rating scale allows us to relate results from the two methodologies.

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## SUBSPECIES CHARACTERIZATION OF UREASE-POSITIVE THERMOPHILIC *CAMPYLOBACTER* (UPTC) ISOLATED FROM SHELLFISH EMPLOYING MODIFIED FLAGELLIN (*flaA*) RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) TYPING

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**ABSTRACT** Shellfish including oysters (*Crassostrea gigas*), cockles (*Cerastoderma edule*) and mussels (*Mytilus edulis*), have previously been described as an important source of thermophilic campylobacters, with the potential of causing acute bacterial gastroenteritis in humans. Previous genotyping studies employing the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) typing, based on the flagellin (*flaA*) gene have been unable to generate an amplicon for the urease-positive thermophilic *Campylobacter* (UPTC), which are the predominant taxa associated with shellfish, largely caused by sequence diversity between the UPTC group and *C. jejuni*. Hence the aim of this study was to develop a modified PCR-RFLP genotyping assay, employing polymorphisms within the flagellin (*flaA*) gene of UPTC organisms, which would now allow the successful amplification and typing of previously nontypable UPTC isolates obtained from natural marine environments. A novel primer pair (UPTC *flaF*/UPTC *flaR*) was designed based on conserved regions within the *flaA* gene locus of UPTC organisms to generate a 1,358 bp amplicon for all UPTC organisms tested. RFLP analysis with *DdeI* in combination with computational analysis of genetic relatedness using BioNumerics software demonstrated the presence of four distinct *flaA* genotypes, among the seven UPTC isolates. In conclusion, this study describes a PCR-RFLP method, based on modified primers from UPTC *flaA* gene sequences that may be successfully applied to examine subspecies relatedness of UPTC organisms from natural environments, including shellfish.

**KEY WORDS:** *Campylobacter jejuni*, DNA, flagellin (*flaA*), genotyping, PCR, shellfish, urease-positive thermophilic *Campylobacter* (UPTC)

### INTRODUCTION

Thermophilic *Campylobacter* spp., including *C. jejuni*, *C. coli* and *C. lari*, are the most common cause of acute bacterial gastroenteritis in the developed Western world (Altekruse & Tollefson 2003). In Northern Ireland, laboratory confirmed isolates account for an approximate annual total of 800–1,000 cases, representing an attack rate of 59 cases per 100,000 individuals (Anon. 2002). In general, most *Campylobacter* infections are believed to be transmitted zoonotically to humans from various animal reservoirs, including poultry, cattle, pigs and from household pets, including cats and dogs, although the epidemiology and routes of transmission are still not completely understood. In addition, various reports have described the presence of thermophilic *Campylobacter* in shellfish, including oysters (*Crassostrea gigas*), cockles (*Cerastoderma edule*) and mussels (*Mytilus edulis*) (Wilson & Moore 1996, Endtz et al. 1997). Given that shellfish may be cultured and harvested in marine waters that are contaminated with agricultural run-off or with human sewage, shellfish concentrate fecal pathogens, including campylobacters

mainly in their gill tissues and such produce may be a hazard to public health, particularly if (1) they are consumed raw; (2) are eaten following only a partial cooking or (3) are allowed to cross-contaminate other foods and utensils in the kitchen, where they are being prepared.

Various previous studies have demonstrated problems in the genotyping of the most common form of *Campylobacter* in shellfish, mainly the urease-positive thermophilic *Campylobacter* (UPTC) (Moore et al. 2003, Sekizuka et al. 2004), whereby these isolates failed to be typed using conventional flagellin (*flaA*)-based restriction fragment length polymorphism (RFLP) analysis, as described originally by Nachamkin et al. (1993). This inability to generate a PCR amplicon with the UPTC organisms was most likely to be caused by significant mutations in the flagellin gene structure in the UPTC organisms, compared with *C. jejuni*, from where the PCR primers were originally designed.

Hence, it was the aim of this study to develop a modified PCR-RFLP genotyping assay, using polymorphisms within the flagellin (*flaA*) gene of UPTC organisms as an epidemiological marker, which would now allow the successful amplification and typing of previously nontypable UPTC isolates obtained from shellfish and environmental water sources.

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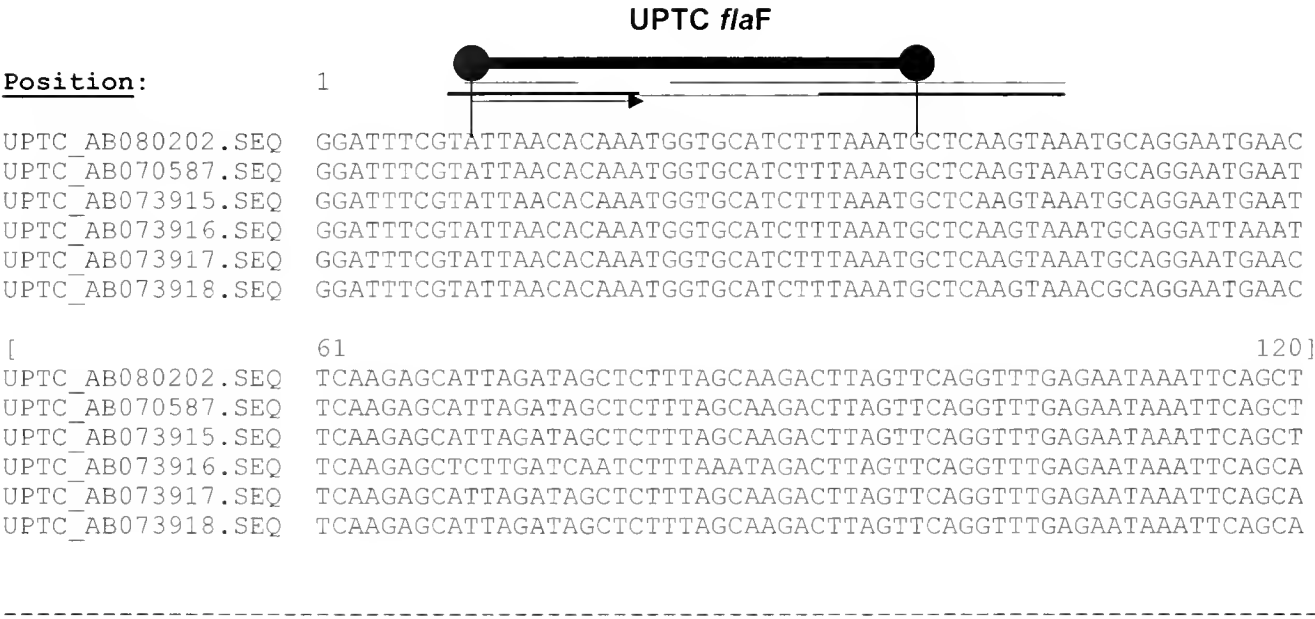
MATERIALS AND METHODS

Design of Modified *flaA* Primers

Two novel PCR primers were designed and designated as UPTC *flaF*(forward primer) and UPTC *flaR* (reverse). The *flaA* gene sequences of six UPTC isolates, which had been recently

published, including UPTC AB07058, AB073915, AB073916, AB073917, AB073918 and AB080202 (GenBank Accession number), were aligned employing the Clustal alignment tool in combination with DNASTAR software (DNASTAR Inc, Wisconsin, USA). Conserved sites were selected (Fig. 1) and contained the following sequences UPTC *flaF* 5'-ATT AAC ACA AAT GGT

(a). Position of UPTC *flaF* (forward primer)



(b). Position of UPTC *flaR* (reverse primer)

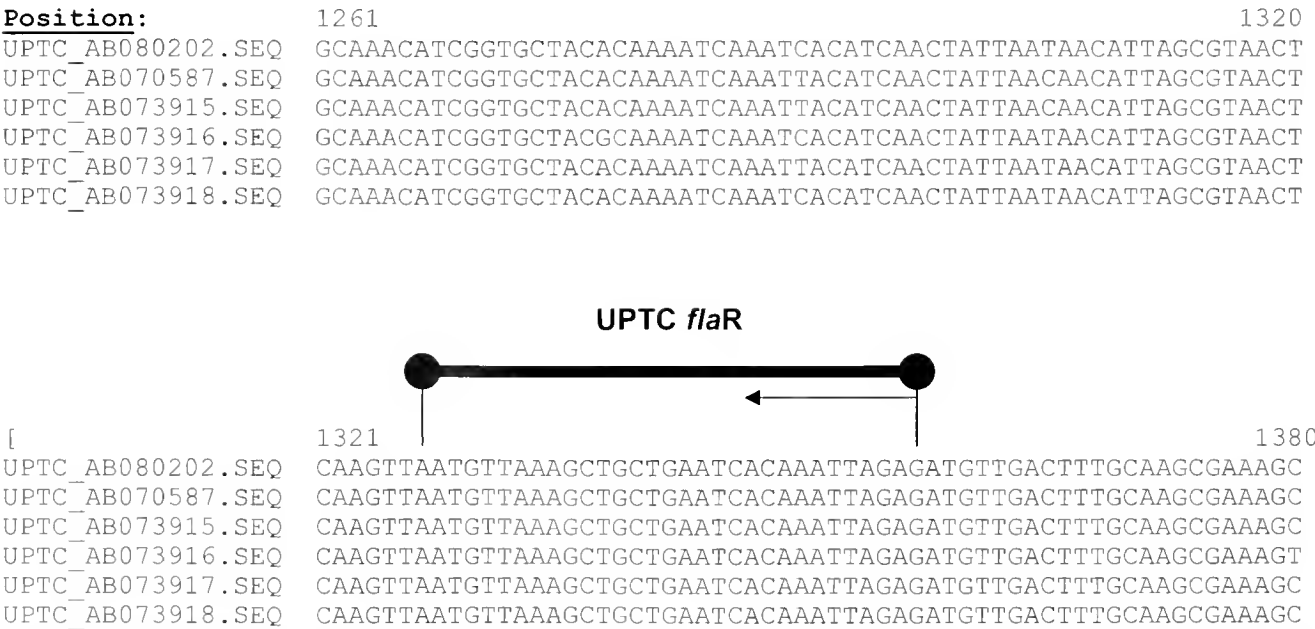


Figure 1. Alignment of the flagellin (*flaA*) gene locus in six isolates of urease-positive thermophilic *Campylobacter* (UPTC) showing (a) conserved nature of forward primer (UPTC *flaF*) and (b) conserved nature of reverse primer (UPTC *flaR*). UPTC isolates detail their GenBank accession numbers.



GCA TCT TTA AAT G-3', corresponding to base positions 10–37 in relation to UPTC AB080202 and UPTC *flaR* 5'-CTC TAA TTT GTG ATT CAG CAG CTT TAA CAT T-3', corresponding to base positions 1357–1327 in relation to UPTC AB080202. Amplification using these primers was estimated to generate a PCR amplicon of approximately 1,358 bp in length.

#### Source of UPTC Isolates Used in This Study

Seven isolates belonging to the UPTC biovar of *C. lari* were used in this study, including four wildtype isolates and three reference isolates, obtained from different environments and in different countries, as detailed in Table 1. In addition, *C. jejuni* ATCC33560 was also used in this study.

#### DNA Extraction

All isolates were cultured under microaerophilic conditions (5% v/v CO<sub>2</sub>) for 48 h on Butzler selective medium, and their species designation was confirmed, as described previously (Moore et al. 2003). Bacterial genomic DNA was extracted in accordance with the method of Harrington et al. (1999) and resulting nucleic acid (DNA) was quantified and adjusted to yield a working concentration of approximately 1.0 µg/µL. Extracted DNA was stored frozen at -20°C until used and at 4°C thereafter.

#### PCR Amplification and Restriction Fragment Length Polymorphism (RFLP) Analysis

After PCR optimization, amplification was performed on all seven UPTC isolates, as well as with the *C. jejuni* isolate, as detailed in Table 1. Reaction mixes (25 µL) were set up as follows: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 200 µM (each) dATP, dCTP, dGTP and dTTP; 1.25U of *Taq* DNA polymerase (Takara Corp., Tokyo, Japan), 10 pmol (each) of the forward and reverse primers (i.e., UPTC *flaF*/UPTC *flaR*) as described above and 1 µL of DNA template. The reaction mixtures after a "hot start" were subjected to the following optimized thermal cycling parameters in a Perkin Elmer 9700 thermocycler: 96°C for 3 min followed by 40 cycles of 96°C for 1 min, 58.5°C for 1 min, 72°C for 2.5 min, followed by a final extension at 72°C for 10 min. During each run molecular grade water was included randomly as negative controls. After amplification, aliquots (15 µL) were removed from each reaction mixture and examined by electrophoresis (100 V, 60 min) in gels composed of 1.0% (w/v) agarose (L03, Takara Corp., Japan) in 0.5 × TBE buffer, stained with ethidium bromide (5 µg/100 mL). Gels were visualized under UV illumination using a gel image analysis system (Atta Image Saver, Atta Corp., Japan).

TABLE 1.

Isolate Reference	<i>Campylobacter</i> Organism	Source	Country
15	UPTC*	Mussel	N. Ireland
145	UPTC	Mussel	N. Ireland
182	UPTC	Sea water	N. Ireland
CF89-12	UPTC	River water	Japan
NCTC12892	UPTC	River water	England
NCTC12895	UPTC	Mussel	England
NCTC12896	UPTC	Mussel	England
ATCC33560	<i>C. jejuni</i>	Bovine	USA

\* UPTC, urease-positive thermophilic *Campylobacter*.

A restriction map was prepared from analysis of the 1,358 bp *flaA* fragment to estimate the optimal restriction enzymes(s) to use to detect polymorphisms in this fragment, using MapDraw software (DNASar Inc., Wisconsin, USA). After PCR amplification, resulting amplicons (10 µL) were digested to completion with the restriction endonuclease *Dde* I (10U) (cleavage site sequence CTGCA↓G; Toboyo Co. Ltd., Osaka, Japan), at 37°C for 4 h in the manufacturer's (H) buffer at the recommended concentration. Digested DNA fragments (15 µL) from the *Dde* I digestion were subjected to electrophoresis, as described earlier, with the exception that digested fragments were separated on a 2.5% (w/v) agarose gel and visualized, as detailed earlier.

#### Computer Estimation of Genetic Relatedness

Computer analysis of the RFLP banding patterns obtained was performed with the BioNumerics software package (Applied Maths, Kortrijk, Belgium). Initially, all RFLP banding patterns were normalized and all images were compatible with one another after normalization, and complete RFLP patterns were used for analysis. In general, bands were automatically assigned by the computer and were corrected manually after the original images were visually checked. Only clearly resolved bands were counted. The Pearson coefficient was used to analyze the similarities of the banding patterns. The unweighted pair group method with average linkages (UPGAMA) was used for cluster analysis and the cophenetic correlation coefficient for the whole dendrogram was calculated for estimation of the faithfulness of the cluster analysis, using the BioNumerics software.

## RESULTS AND DISCUSSION

PCR amplification using the novel primer pair, UPTC *flaF*/UPTC *flaR*, allowed the reproducible generation of a *flaA* amplicon of approximately the expected size (*circa* 1,358 bp) for all UPTC isolates examined (Fig. 2). A slightly larger amplicon (*circa* 1,500 bp) was generated for *C. jejuni* ATCC33560 (data not shown). Restriction map analysis with *Dde*I demonstrated the presence of at least five restriction sites within the PCR amplicon, yielding fragments at positions 82, 155, 644, 885 and 910. Subsequent restriction of PCR amplicons demonstrated the presence of at least three bands, which were grouped into four banding profiles (genotypes), as determined by analysis by the BioNumerics software (Fig. 3).

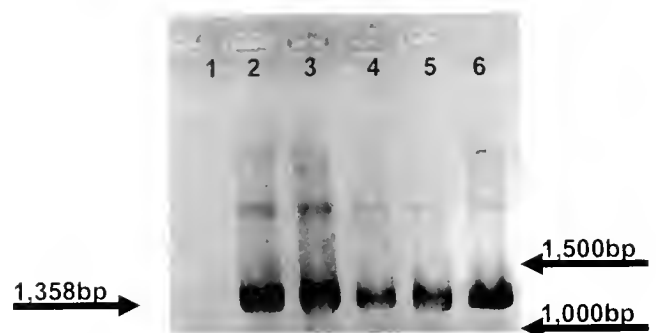


Figure 2. PCR amplification of a 1,358 bp fragment of the *flaA* gene of urease-positive thermophilic *Campylobacter*. Lane 1, negative control (molecular grade water), lane 2, UPTC 15; lane 3, UPTC 145, lane 4, UPTC 182, lane 5, UPTC CF89-12 & lane 6, UPTC NCTC12892. UPTC, urease-positive thermophilic *Campylobacter*.

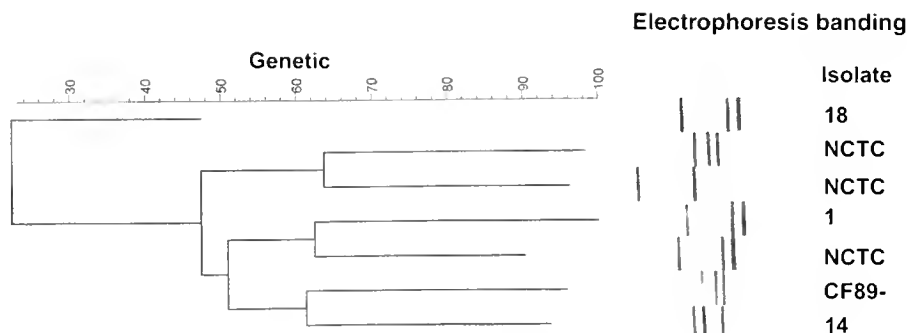


Figure 3. Dendrogram derived from RFLP analysis with UPTC *flaF*/UPTC *flaR* primers targeting a 1,358 bp fragment of the flagellin (*flaA*) gene in seven isolates of urease-positive thermophilic *Campylobacter* (UPTC) (see Table 1). *flaA* PCR amplicons were digested to completion with *DdeI* restriction enzyme. The Pearson coefficient was used to calculate similarities and UPGAMA was used for cluster analysis with BioNumerics software (Applied Maths, Belgium).

Marine bivalve molluscs such as mussels (*Mytilus edulis*), cockles (*Laevicardium edula*), scallops (*Pecten maximus*) and oysters (*Crassostrea gigas*) are grown commercially in in-shore waters around Northern Ireland with an annual production and market value of 288.4 tons and £527,733 respectively. These shellfish feed by filtering plankton and detritus from large volumes of sea water, thereby concentrating gastrointestinal pathogenic organisms found in such waters, including *Campylobacter*, *Salmonella* and enteric viruses in their digestive system (Wilson & Moore 1996), which may be important vectors in the transmission of disease. The association between ingestion of raw shellfish and enteric diseases such as typhoid has been recognized and more recently, there has been several reports of shellfish-associated gastroenteritis with strong evidence for *Campylobacter* spp. as the etiologic agent. *Campylobacter* spp. have been found routinely in West Coast shellfish beds in the States of California, Oregon and Washington (Abeyta et al. 1993). Sample types included oysters, cockles, sediment, seawater, freshwater (streams and tributaries), bird excreta and cattle manure. *Campylobacter* spp. in the marine environment primarily comes from wild birds (Kapperud & Rosef 1983), farm runoff, surface water (Carter et al. 1987) and sewage bypasses (Jones 2001).

Urease-positive thermophilic *Campylobacter* (UPTC), a microaerophilic and Gram-negative bacterium, is an organism only identified relatively recently in England (Bolton et al. 1985). After the original descriptions of UPTC appeared, isolates of UPTC were reported in France, Ireland and The Netherlands, and UPTC strains have also recently been found in Japan (Matsuda et al. 1996). The original strains were isolated from river water, sea water, mussels and cockles. In 1988, Megraud et al. reported the first isolation of UPTC from human clinical infection, where these organisms were isolated from an appendix as well as from human faeces (Megraud et al. 1988).

This group of organisms has been frequently found as the most frequent *Campylobacter* found in shellfish and waters (Wilson & Moore 1996).

The understanding of sources and means of transmission of UPTC organisms is important to help elucidate the epidemiology of these organisms in shellfish and the marine environment, as well as their potential transmission to humans. Consequently a reproducible, sensitive and well-standardized typing scheme is critical in the successful discrimination of such strains. Hence to elucidate the sources and modes of transmission of these organisms, it is

essential to use epidemiological typing methods that discriminate between different strains but which are reliable and reproducible in the recognition of similar strains.

This study was interested in the ability to genotype members of the UPTC group of organisms isolated in shellfish and was undertaken to develop a PCR-based method that would allow the differentiation between UPTC organisms at the subspecies level. Until now, employing the conventional PCR-RFLP (*flaA*) method, as described originally for the subspecies differentiation of clinical *C. jejuni*, has not been successful in its application with the UPTC organisms (Moore et al. 2003). Furthermore, because this method is widely used for other campylobacters, it was important to develop it further, so that it may be used successfully with this group of organisms. Previous inability to generate a PCR amplicon using the Nachamkin *flaA* primers suggested that the UPTC organisms had an altered flagellin gene arrangement compared with *C. jejuni*, with various mutations at the original primer sites. Subsequently, we have previously reported this altered flagellin gene rearrangements in UPTC, through the sequencing and analyses of the *flaA* gene of several UPTC wildtype and reference strains (Sekizuka et al. 2004). Hence, it was the aim of the current study to use this sequence data from UPTC organisms and design a modified assay that would allow the successful amplification of these organisms, helping facilitate their subsequent subspecies analyses.

After the development of a successful and reproducible PCR-RFLP system, employing the newly described primer pair (UPTC *flaF*/UPTC *flaR*), we wished to establish proof-of-principle for this technique in demonstrating the application of this method to a small collection of UPTC isolates obtained from the natural environment. Application of this technique to this small collection of isolates demonstrated genetic variability at the *flaA* locus between these isolates (Fig. 3). Shellfish growing in relatively shallow marine waters, including inshore waters, may be contaminated with campylobacters from several sources, in particular, effluent from agricultural run-off and human sewage. In addition, they may become contaminated because of fecal deposition of UPTC from wild birds, including members of the gull (*Larus*) family. In this study, genotypic analysis demonstrated the presence of the same genotype of UPTC in shellfish isolated from the natural environment, located approximately 10–30 kilometers from each other, with no sharing of the same watertable. This may suggest that gulls may have been responsible from the transmission of the same

genotype of UPTC between the two sites and hence acted as a vector of transmission of this particular genotype.

In conclusion, this study describes a PCR-RFLP method, based on modified primers from UPTC *flaA* gene sequences, that may be successfully applied to examine subspecies relatedness and routes of transmission of UPTC isolated from shellfish, so that we are better informed as to how these become contaminated to suggest intervention controls that would help reduce the loading of these shellfish with campylobacters. Further work is now required to establish routes of transmission of UPTC organisms from the environment to shellfish employing this modified technique.

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## THE EFFECTIVENESS OF HEAT, COLD AND 6-DIMETHYLAMINOPURINE SHOCKS FOR INDUCING TETRAPLOIDY IN THE KURUMA SHRIMP, *MARSUPENAEUS JAPONICUS* (BATE)

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**ABSTRACT** In this study tetraploid *Marsupenaeus japonicus* (Bate) embryos were produced by preventing the first division in mitosis. The effectiveness of temperature and chemical shocks for producing tetraploid *M. japonicus* were assessed when applied at different times postspawning and for different durations. Tetraploid *M. japonicus* embryos (spawned at 27°C) were produced by heat shocks at 35°C and 36°C in three and eight spawning samples respectively, and a cold shock at 5°C in a single spawning sample. All temperature shocks inducing tetraploidy were applied 18–23 min postspawning for a 5–10 min duration. The percentage of spawnings successfully inducing tetraploid embryos (i.e., frequency of induction) ranged from 33.33% to 66.67% for the 21, 22 and 23 min postspawning heat shock treatment regimes. The percentage of tetraploid embryos within an induction (i.e., induction rate), as determined by flow cytometry, ranged from 8.82% to 98.12% (ave.  $\pm$  S.E.) ( $34.4 \pm 21.4\%$ ) for the 35°C shock treatments, from 13.12% to 61.02% ( $35.0 \pm 5.0\%$ ) for the 36°C shock treatments and was 15% for the 5°C cold shock treatment. No tetraploids were produced for spawnings that received heat shocks above 36°C or below 35°C, or for cold shocks above 5°C for any of the tested postspawning treatment and duration times. Chemical shock with 150  $\mu$ M 6-dimethylaminopurine did not result in tetraploid *M. japonicus* embryos at any of the tested postspawning treatment times and durations. Tetraploid *M. japonicus* embryos were nonviable, with no tetraploid larvae being detected by flow cytometry. Based on our results heat shocking of *M. japonicus* embryos at 36°C, 23 min postspawning for a 5–10 min duration is the most effective means to produce tetraploids through inhibition of the first mitotic division (taking into consideration the importance of frequency and induction rate equally).

**KEY WORDS:** *Penaeus japonicus*, polyploidy, shrimp, selective breeding, genetic protection

### INTRODUCTION

In Australia, shrimp domestication and genetic improvement programs are most advanced for *Marsupenaeus japonicus* (Bate) (Preston et al. 2001). The development of selectively improved genotypes for over 10 generations and a live export market has prompted industry to seek mechanisms to sterilize stocks to prevent unlicensed breeding and the introduction of genetically improved strains into natural fisheries. Previous efforts to contain selectively bred *M. japonicus* have focused on inducing sterility via irradiation or triploidy. These techniques have yet to prove effective in providing guaranteed sterility in the target stocks. Irradiation has been reported to significantly impair the reproductive capacity of female *M. japonicus* when exposed to 20 gray and male *M. japonicus* when exposed to 10 gray (Sellars et al. 2005b). However, irradiation was not 100% effective at preventing the production of viable offspring. In comparison, successful triploidy induction through prevention of polar body II extrusion is 100% effective at preventing reproduction, however, inductions never result in 100% triploid progeny (Sellars et al. 2004, Norris et al. 2005).

Producing triploids by the mating of tetraploids with diploids may provide a solution to these variable induction rates, resulting in 100% triploid progeny. Triploid stocks have been produced through the mating of tetraploid and diploid broodstock in several marine species including Pacific oysters (*Crassostrea gigas*) (Guo & Allen 1994, Guo et al. 1996, Wang et al. 2002), oyster hybrids (*C. gigas*  $\times$  *C. ariakensis*) (Huayong & Allen 2002), Rainbow trout (*Oncorhynchus mykiss*) (Chourrout et al. 1986) and carp hybrids (*Crassius auratus* red var.  $\times$  *Cyprinus carpio* L.) (Liu et al. 2001).

However, production of viable tetraploids and their successful mating with diploids is a prerequisite for producing triploids in this manner.

There are many different methods for inducing tetraploidy in aquatic species, with the inhibition of an early embryonic developmental phase being critical to all. Because triploid *M. japonicus* do not produce viable gametes (Sellars et al. 2003, Preston et al. 2004, Sellars et al. 2004), there are only two possible ways to induce tetraploidy in this species: inhibition of polar body I extrusion during meiosis and prevention of the first division in mitosis. The prevention of polar body I extrusion has been reported to complicate subsequent chromosome segregation (Guo et al. 1992), and result in many different ploidy combinations including viable tetraploids. Inhibition of polar body I extrusion is difficult in some species because of the very short time frame in which the embryos must be treated and the fragile nature of the newly spawned embryos. In *M. japonicus* polar body I extrusion occurs at 4 min 10 sec postspawning at 27°C (Hudinaga 1941). Successful detection, collection, concentration and application of a shock to spawned embryos within this short time frame is a significant challenge. Only one reported study has attempted preventing polar body I extrusion in shrimp, however no tetraploids were successfully induced (Li et al. 2003).

Preventing the first division in mitosis is the alternative strategy that has been the focus of several previous attempts to induce tetraploidy in shrimp (Xiang et al. 1993, Peeters 1996, Li et al. 2003, Deoraj et al. 2005). Viable tetraploid shrimp, produced by inhibition of the first mitotic division, have only been reported in a single study by Xiang et al. (1993) in *Ferropenaeus* (*Penaeus*) *chinensis* using chemical and temperature shocks. Deoraj et al. (2005) reported poor viability of tetraploid and polyploid *Litopenaeus vannamei* embryos induced through heat shock, whereas Peeters (1996) reported unsuccessful tetraploid inductions in *Pe-*

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*naeus indicus* using chemical shocks. Li et al. (2003) reported successful induction of tetraploid *F. chinensis* embryos, however the larvae were not viable. Notably, all reported tetraploid inductions in shrimp have used temperature or chemical shocks (Xiang et al. 1993, Peeters 1996, Li et al. 2003, Deoraj et al. 2005).

This study aimed to assess the effectiveness of different temperature and chemical shock agents to produce tetraploid *M. japonicus* by preventing the first division in mitosis, which begins at 30 min postspawning at 27°C (Hudinaga 1941). A number of treatments were tested at various times after spawning and for different durations to determine which treatment regimes could be used to produce tetraploid *M. japonicus*.

## MATERIALS AND METHODS

### Source of Broodstock and Maturation

Wild *M. japonicus* broodstock were captured from coastal waters near Mackay, Queensland, Australia (21°09'S, 149°30'E) by trawling at a depth of approximately 120 m. Broodstock were maintained (25 females: 15 males) in 2,000-L round fiberglass tanks, each fitted with a subsand circulation system (Crococ & Coman 1997). Tanks received 1.6 L min<sup>-1</sup> of 10-µm filtered, 34 ppt salinity seawater at 27 ± 2°C and were maintained on a 12 h light:12 h dark cycle. Shrimp were fed commercial *M. japonicus* pellets *ad libitum* once per day and fresh squid (*Loligo spp.*) three times a week during dark hours.

Ovarian development was assessed by shining a torch beam through the dorsal exoskeleton of the females during dark hours (Crococ & Coman 1997). Impregnated females that were ready to spawn (stage IV; Crococ & Kerr 1983) were caught, unilaterally eye-stalk ablated using hot forceps, and transferred to 100-L circular spawning tanks filled to 40 L. Spawning tanks received 0.2 L min<sup>-1</sup> of 10-µm filtered, 34 ppt salinity seawater at 27 ± 2°C and were maintained on a 12-h light:12-h dark cycle. Females in spawning tanks were fed one piece (2 cm<sup>3</sup>) of fresh chopped squid (*Loligo spp.*) daily during light hours.

### Spawning Detection and Embryo Collection

Spawning tanks were fitted with an automated spawning detection system (Coman et al. 2003) that accurately detects spawnings of *M. japonicus* within 2 min of spawning. The time of alarm from the automated detection system was taken as time zero and referred to from hereon as time postspawning. For ease of explanation spawned eggs, whether fertilized or unfertilized, will be referred to as embryos from hereon. At 10 min postspawning embryos were siphoned onto a 60-µm screen suspended in a 1-L beaker of seawater. Seawater was allowed to flow out of the beaker, resulting in the concentration of embryos on the screen. Once embryos were concentrated (approx. 60,000–100,000 embryos L<sup>-1</sup>), the screen was lifted out of the beaker and embryos were rinsed into a beaker containing between 200–800 mL of seawater depending on the experimental design for that spawning. All seawater used for embryo collection, hatching and chemical stock solution preparation was 10-µm filtered and maintained at 27 ± 2°C and 34 ppt salinity.

### Induction and Hatching of Collected Embryos

Initial experiments using heat and cold shocks were conducted to assess a range of temperatures for 3 durations (5, 10 and 15 min). Once a suitable temperature shock was established for in-

ducing tetraploidy (based on consistent inductions at the different durations), we further assessed different postspawning treatment times using the 5- and 10-min treatment durations. For the chemical shock experiments a predetermined treatment concentration was chosen based on results of *M. japonicus* triploid induction studies (Norris et al. 2005), which inhibited early embryonic processes. Initial experiments using chemical shock were therefore focused at assessing different postspawning treatment times for variable treatment durations (5–15 min).

Tetraploid inductions were timed to prevent the first division in mitosis. Various shock agents were trialed including heat, cold and chemical shocks. Shocks were applied between 16–28 min postspawning, with durations varying from 5–15 min. Control embryos were included in each experiment or spawning sample and received the same handling stress as their treated siblings. Control and treatment embryos from all spawnings were incubated at 27 ± 2°C with light aeration until hatching. Notably, only spawnings with hatching rates of >40% in the controls were used in this study. Depending on the quantity of eggs spawned and number of available people to complete inductions, in some instances spawnings were divided into two or three spawning samples.

### Heat Shock Inductions

Heat shock inductions were completed on 26 spawning samples in total. Heat shocks varied from 32°C to 46°C and were applied from 18–24 min postspawning for a 5, 10 or 15 min duration (Table 1). A similar technique as described by Sellars et al. (2005a) to expose *M. japonicus* embryos to ozone was used to expose embryos to heat shocks. Briefly, concentrated embryos were divided into enough 100-mL aliquots so that there was one for each postspawning treatment time. These aliquots were further divided into enough subaliquots so that there was one for each treatment duration. At the correct postspawning treatment time each subaliquot of embryos was poured through a 60-µm screen, which was placed directly into a seawater bath at the specified heat shock temperature. Each 60-µm screen was removed from the seawater bath after the treatment duration time had lapsed and embryos were rinsed into 200 mL of 27°C seawater. Before, during and after treatment, seawater bath temperature was monitored using a mercury thermometer.

### Cold Shock Inductions

Cold shock inductions were completed on 31 samples from different spawnings in total. Cold shocks ranged from 5°C to 23°C and were applied from 16–28 min postspawning for a duration of 5–15 min (Table 2). For samples of spawnings where there was only one postspawning and duration time, treatment was applied by the addition of cold seawater to concentrated embryos. For these spawnings cold shock was stopped by decanting off the cold water once embryos had settled during the shock treatment, followed by the addition of 27°C seawater at the correct time to give the different treatment durations. When a spawning sample was exposed to a cold shock treatment that had different postspawning or duration times, the same procedures as outlined earlier for heat shock inductions were used.

### Chemical Shock Inductions

Chemical inductions using a final concentration of 150-µM 6-dimethylaminopurine (6-DMAP) (Sigma) were completed on 14 spawning samples in total. Chemical shock was applied between 18–28 min postspawning for a duration of 5–15 min (Table 3).

TABLE 1.

Heat shock treatments applied to different spawning samples to attempt the prevention of the first division in mitosis in *Marsupenaeus japonicus* to induce tetraploidy. Durations with an asterisk (\*) had three replicate treatments.

Heat Shock	Treatment Time		Spawning Sample
	Postspawning (min)	Duration (min)	
32 C	18	5, 10 & 15	1 to 4
	20	5, 10 & 15	1 to 4
	22	5, 10 & 15	1 to 4
	24	5, 10 & 15	1 to 4
35 C	21	5 & 10	10 to 12
	22	5 & 10	10 to 12
	22	5* & 10	21 to 23
	23	5 & 10	10 to 12
36 C	18	5, 10 & 15	5
	20	5, 10 & 15	5
	21	5 & 10	13 to 15
	22	5, 10 & 15	5
	22	5 & 10	13 to 15
	22	5 & 10	16 to 19
	22	5* & 10*	24 to 26
	23	5 & 10	13 to 15
38 C	22	5 & 10	20
	24	5, 10 & 15	5
39 C	18	5, 10 & 15	6 to 8
	20	5, 10 & 15	6 to 8
	22	5, 10 & 15	6 to 8
	24	5, 10 & 15	6 to 8
46 C	18	5, 10 & 15	9
	20	5, 10 & 15	9
	22	5, 10 & 15	9
	24	5, 10 & 15	9

6-DMAP was applied to embryos from spawning samples 58–63 using the same procedures outlined by Norris et al. (2005). In brief, 28 mL of a 1-mM stock solution of 6-DMAP was added to 160 mL of seawater containing the embryos. For spawning samples 64–71 a similar technique as described for temperature treatment earlier and by Sellars et al. (2005a) to expose *M. japonicus* embryos to ozone was used to expose embryos to 6-DMAP. Briefly, concentrated embryos were divided into 4 × 100-mL aliquots (one for each of the four postspawning treatment times) to which 50 mL of a fresh 450 µM 6-DMAP stock solution (made up in seawater) was added at the correct postspawning treatment time. Each aliquot was then divided into 3 × 50-mL subaliquots. Subaliquots were poured through a 60-µm screen after the appropriate treatment duration to collect the embryos, which were then transferred to 200 mL of seawater to cease chemical exposure.

#### Ploidy Detection and Analysis

Pooled samples of between 5–30 nauplii (less than 30 nauplii were sampled only in instances when less than this hatched) and 50–100 embryos were separately sampled for each spawning from all controls and treatments. In treatments where there were no nauplii hatched, it was only possible to take an unhatched embryo sample. In some instances samples were snap frozen in liquid

TABLE 2.

Cold shock treatments applied to different spawning samples to attempt the prevention of the first division in mitosis in *Marsupenaeus japonicus* to induce tetraploidy.

Cold Shock	Treatment Time		Spawning Sample
	Postspawning (min)	Duration (min)	
5 C	20	8	40
6.5 C	22	5 & 10	54 to 57
7 C	16	5 & 10	42
	25	5	29
	28	7	34 & 35
	16	10	45
8 C	20	8	39
	18	5, 10 & 15	46 to 49
	20	5, 10 & 15	46 to 49
	21	5 & 10	50 to 53
9 C	22	5, 10 & 15	46 to 49
	22	5 & 10	50 to 53
	23	5 & 10	50 to 53
	24	5, 10 & 15	46 to 49
9.5 C	16	5 & 10	41
	16	10	44
10 C	16	10	43
11 C	20	8	38
12 C	28	7	32 & 33
14 C	20	8	37
17 C	25	5	28
	28	7	30 & 31
22 C	25	5	27
23 C	20	8	36

TABLE 3.

150 µM 6-dimethylaminopurine shock treatments applied to different spawning samples to attempt the prevention of the first division in mitosis in *Marsupenaeus japonicus* to induce tetraploidy. Durations with an asterisk (\*) had three replicate treatments.

Treatment Time		
Postspawning (min)	Duration (min)	Spawning Sample
18	5, 10 & 15	64 to 71
20	10	59
20	8	60 & 61
20	6	62 & 63
20	5, 10 & 15	64 to 71
22	10	59
22	8	60 & 61
22	6	62 & 63
22	5, 10 & 15	64 to 71
24	5, 10 & 15	64 to 71
25	10	59
25	8	60 & 61
25	6	62 & 63
26	10	59
26	8	60 & 61
26	6	62 & 63
28	10	58

nitrogen and stored at  $-20^{\circ}\text{C}$  for up to 30 days. If polyploidy analysis could be completed on the day of hatching, nauplii were sampled and taken live to the laboratory and embryos were transported on ice. Snap frozen samples were transported to the laboratory and defrosted on ice. Live nauplii were chilled on ice at the laboratory until they died. Once dead nauplii or embryos had settled, excess seawater was removed,  $<200\ \mu\text{L}$  of seawater and sample remained in each tube.

At the laboratory  $500\ \mu\text{L}$  of marine phosphate buffered solution (MPBS) ( $11.0\ \text{g L}^{-1}\ \text{NaCl}$ ,  $0.2\ \text{g L}^{-1}\ \text{KCl}$ ,  $1.15\ \text{g L}^{-1}\ \text{Na}_2\text{HPO}_4\cdot 2\text{H}_2\text{O}$ ) propidium iodide (PI) stain (MPBS containing  $0.1\%$  Triton X-100,  $0.2\ \text{mg mL}^{-1}\ \text{Rnase A}$ ,  $0.02\ \text{mg mL}^{-1}\ \text{PI}$ ) was added to each sample. Samples were then homogenated individually by aspiration eight times through a 25-G needle pushed firmly against the side of the sample tube. After homogenation  $10\ \mu\text{L}$  of a 1:100 dilution of the internal standard, glutaraldehyde fixed, chicken red blood cells (CRBC, Handbook of Flow Cytometry

Methods) was added to each sample. Cell suspensions were screened through  $62\text{-}\mu\text{m}$  mesh prior to fluorescent activated cell sorting (FACS) on a Calibur Flow Cytometer (Beckton Dickinson Immunocytometry Systems San Jose, California, USA). A total of 30,000 shrimp cells were analyzed for each sample, however, in some instances only 15,000 shrimp cells were analyzed because there were too few cells in a sample.

Induction rates were calculated for each treatment sample. Initially the proportion of diploid cells in the G2 phase relative to the G1 phase was calculated from a control spawning sample of the same life-history stage. This proportion was used to calculate the number of diploid G2 cells in treatment samples relative to the number of diploid G1 cells in the treatment sample. Once the total number of diploid G2 cells in treatment samples was estimated, the tetraploid G1 peak was calculated by subtracting the diploid G2 estimation from the total number of cells where the tetraploid G1 peak falls (Fig. 1) (within the cell cycle G1 cells are in the growth

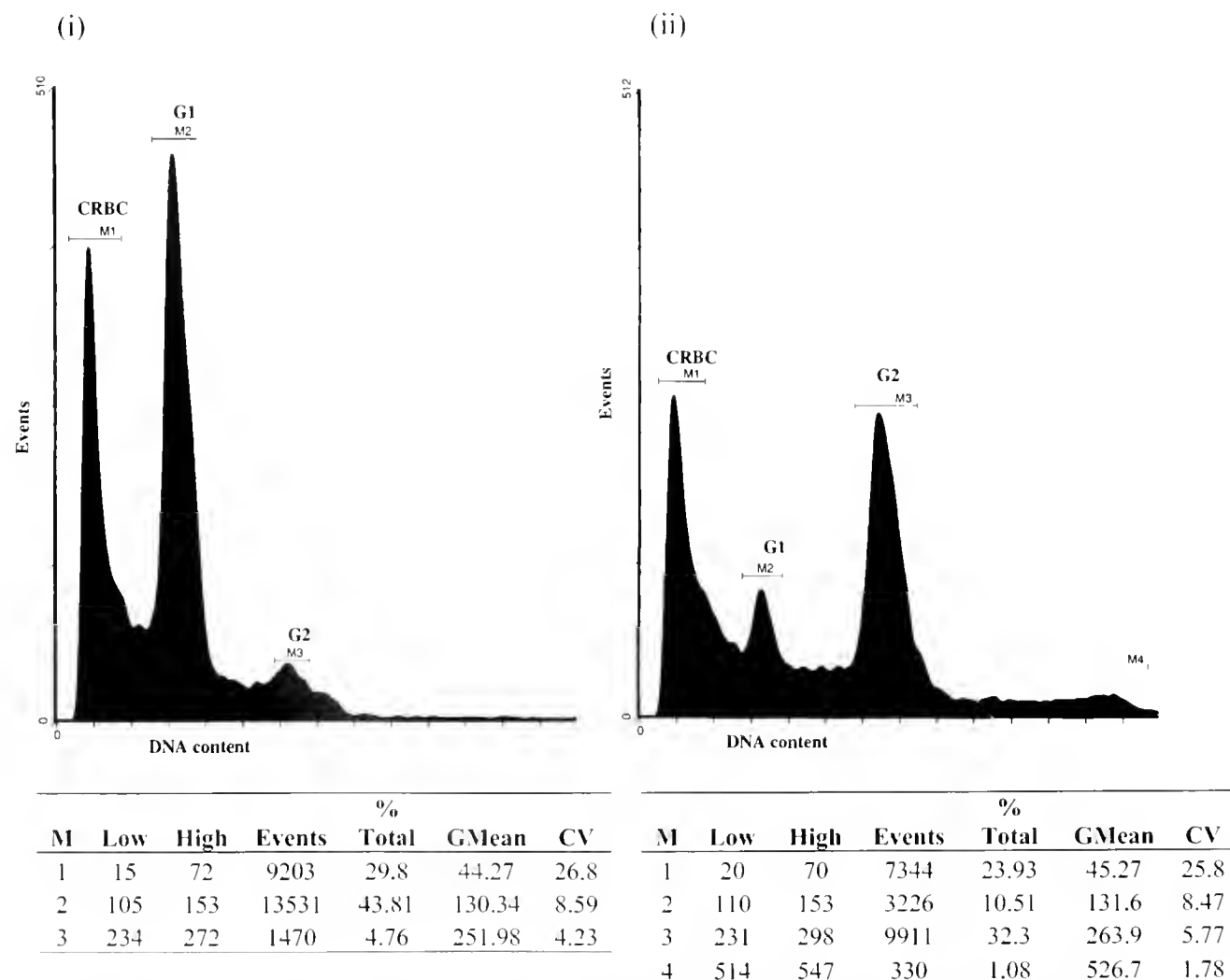


Figure 1. Example of a (i) control and (ii) treatment fluorescent activated cell sorting output from *Marsupenaeus japonicus* embryos. The expected size of the diploid G2 peak in (ii) is calculated from dividing the events in the diploid G2 peak in (i) by the diploid G1 peak in (i), and then multiplying this factor by the events in the diploid G1 peak in (ii). When the total number of events of the G2 peak in (ii) was greater than expected (by 5% or more), ModFit analysis was performed to determine the different levels of polyploidy. In this instance the expected diploid G2 peak in (ii) would be  $(1470/13531) \times 3226 = 350.47$  events. Because the actual number of events with a DNA content the same as a diploid G2 cell in (ii) is 9911, and we would only expect  $\sim 350$  diploid G2 cells, this output would be analyzed by ModFit to determine the tetraploidy level. CRBC is an internal standard control (chicken red blood cells).



phase, whereas G2 cells have double the normal chromosomal content and are preparing for division). Where the number of cells counted with a DNA content the same as a diploid G2 cell was greater than expected (by 5% or more), the level of polyploidy in that sample was analyzed using ModFit software (Verity Software House, Topsham, Maine, USA). A five percent threshold was chosen based on observed variability among multiple samples taken from the same control groups.

The frequency of producing tetraploid embryos was calculated for the trialed heat shock treatment regimes by dividing the number of spawning samples within a treatment regimen, which had tetraploid embryos by the total number of induction attempts for that treatment regimen.

The effect of heat shock treatment (control, 35°C, 36°C) and duration (5 min, 10 min) on induction rates at 22 min postspawning were analyzed by 2-way ANOVA (PROC GLM; SAS Institute Software, 1999) for spawning samples in which one or more of the treatment combinations resulted in tetraploid embryos. Pairwise comparisons between shock and duration treatments were performed using the least significant difference test.

## RESULTS

### Heat Shock Inductions

From the initial scoping experiments it was evident that 35°C and 36°C heat shocks were most suitable for inducing tetraploidy. Tetraploid embryos were produced most frequently by a 35°C heat shock applied 22 min postspawning for a 10 min duration (66.67% of the time) (Table 4). Tetraploid induction rates for this 35°C treatment regimen were 8.82, 20.9, 9.9 and 98.1% (ave.  $\pm$  S.E.) ( $34.4 \pm 21.4\%$ ) for spawning samples 21, 22 and spawning sample 23 replicate 1 and 2 respectively. The frequency of tetraploid embryo induction was lower for the 36°C heat shock treatments ranging from 33.33% to 45.45% for the 21, 22 and 23 min postspawning treatments at the 5 and 10 min duration times trialed (Table 4). Tetraploid induction rates for the 36°C shock treatments ranged from 13.12% to 61.01%, (ave.  $35.0 \pm 5.0\%$ ). Only one 18

min postspawning induction was applied at 36°C for a 10 min duration which did produce tetraploid embryos, resulting in a 100% tetraploid induction frequency (Table 4).

When treated at 22 min postspawning, tetraploid induction rates of embryos were significantly higher ( $P < 0.05$ ) for the 36°C shock applied for a 5 min duration (ave.  $12.81 \pm 5.80\%$ ) compared with the control (27°C) (ave.  $0.00 \pm 0.00\%$ ) and 35°C (ave.  $0.00 \pm 0.00\%$ ) shock treatments (Table 5). However, when the duration of treatment was increased to 10 min, there was no significant difference between tetraploid induction rates for the 35°C and 36°C shock treatments (ave.  $34.43 \pm 21.39$  and  $18.52 \pm 8.53\%$  respectively) and both were significantly higher than controls (27°C) (ave.  $0.00 \pm 0.00\%$ ) (Table 5). Because tetraploid embryos were produced at two of the three duration times trialed for the 36°C shock, compared with only one for the 35°C shock, further experimentation to optimize postspawning treatment times used a 36°C temperature shock.

Although no statistical comparisons were possible as too few spawning samples had tetraploid embryos in the different 36°C shock postspawning treatment times trialed, tetraploid induction rates of embryos were higher on average for the 21, 22 and 23 min postspawning times when the treatment duration was 10 min compared with 5 min (i.e., 61.00%, 18.52% and 53.40% respectively compared with 12.5%, 12.81% and 45.00%) (Table 6). These results suggest that duration had a stronger effect than postspawning time on tetraploid induction.

There were no tetraploid nauplii produced in any of the heat shock treatments trialed. It is also worth noting that all treatment groups for the 39°C and 46°C spawning samples had zero hatch, and FACS outputs of embryos from these treatments were difficult to analyze because the different cell phases could not be discriminated between because of tissue degradation.

### Cold Shock Inductions

Only one of the trialed cold shock treatment regimes produced tetraploid embryos. In this treatment, spawning embryos treated at

TABLE 4.

Frequency of producing tetraploid *Marsupenaeus japonicus* embryos at the different heat shock treatment combinations trialed.

Treatment Time		Heat Shock				
Postspawning (min)	Duration (min)	32°C	35°C	36°C	38°C	39°C
18	5	0% (n = 4)		0% (n = 1)		0% (n = 3)
18	10	0% (n = 4)		100% (n = 1)		0% (n = 3)
18	15	0% (n = 4)		0% (n = 1)		0% (n = 3)
20	5	0% (n = 4)		0% (n = 1)		0% (n = 3)
20	10	0% (n = 4)		0% (n = 1)		0% (n = 3)
20	15	0% (n = 4)		0% (n = 1)		0% (n = 3)
21	5		0% (n = 3)	33.33% (n = 3)		
21	10		0% (n = 3)	33.33% (n = 3)		
22	5	0% (n = 4)	0% (n = 6)	45.45% (n = 11)	0% (n = 1)	0% (n = 3)
22	10	0% (n = 4)	66.67% (n = 6)	45.45% (n = 11)	0% (n = 1)	0% (n = 3)
22	15	0% (n = 4)		0% (n = 1)		0% (n = 3)
23	5		0% (n = 3)	33.33% (n = 3)		
23	10		0% (n = 3)	33.33% (n = 3)		
24	5	0% (n = 4)		0% (n = 1)		0% (n = 3)
24	10	0% (n = 4)		0% (n = 1)		0% (n = 3)
24	15	0% (n = 4)		0% (n = 1)		0% (n = 3)

TABLE 5.

Average tetraploid induction rate ( $\pm$ SE) of *Marsupenaeus japonicus* embryos from spawning samples in which tetraploid embryos were produced in one or more treatment category. Means with different superscripts are significantly different ( $P < 0.05$ ) within duration.

Duration	Temperature Shock Applied 22 min Postspawning		
	27 °C	35 °C	36
5 min	0.00 $\pm$ 0.00% <sup>b</sup> (n = 8)	0.00 $\pm$ 0.00% <sup>b</sup> (n = 4)	12.81 $\pm$ 5.80% <sup>a</sup> (n = 8)
10 min	0.00 $\pm$ 0.00% <sup>b</sup> (n = 8)	34.43 $\pm$ 21.39% <sup>a</sup> (n = 4)	18.52 $\pm$ 8.53% <sup>a</sup> (n = 8)

5°C for 8 min at 20 min postspawning had a tetraploid induction rate of 15.0%. Nauplii sampled from this same treatment were found to be diploid. All other cold shock inductions failed to induce tetraploidy in any of the embryo and nauplii samples. All spawnings on which cold shocks were applied hatched resulting in all control and treatment nauplii FACS samples containing ~30 nauplii.

#### 6-DMAP Shock Inductions

6-DMAP inductions using a 150- $\mu$ M final concentration failed to produce tetraploid *M. japonicus* when applied 18–28 min postspawning, for 5–15 min duration. All embryo and nauplii samples in the control and treatment groups were found to be diploid. All spawnings on which 6-DMAP shocks were applied hatched resulting in all control and treatment nauplii FACS samples containing ~30 nauplii.

#### DISCUSSION

The results of this study demonstrate that heat and cold shock can prevent the first division in mitosis to produce tetraploid *Marsupenaeus japonicus* embryos. These findings are consistent with those of Xiang et al. (1993) and Li et al. (2003) who successfully inhibited the first division in mitosis in *Ferropenaeus* (*Penaeus*) *chinensis* using temperature shocks to produce tetraploid embryos. To our knowledge there are no other reports of successful tetraploid induction in Penaeid shrimp.

Tetraploid induction rates and frequency of induction of *M. japonicus* embryos were highly variable (0% to 98% and 33.33% to 100% respectively). This finding is similar to other polyploidy induction studies that have attempted to prevent an early embry-

TABLE 6.

Tetraploid induction rates of *Marsupenaeus japonicus* embryos when exposed to a 36 °C shock at different postspawning times for a 5 or 10 min duration.

Time Postspawning	Duration	
	5 min	10 min
18 min		32.60% (n = 1)
20 min	0.00 (n = 1)	0.00% (n = 1)
21 min	12.50% (n = 1)	61.00% (n = 1)
22 min	12.81% $\pm$ S.E. 5.80% (n = 8)	18.52% $\pm$ SE 8.53% (n = 8)
23 min	45.00% (n = 1)	53.40% (n = 1)

onic development phase (Xiang et al. 1993, Li et al. 2003, Sellars et al. 2004, Norris et al. 2005). In general, polyploidy induction rates are dependant on three main variables: magnitude of shock (e.g., change in temperature), timing of shock and shock duration. This study showed that increasing temperature by 9°C (from 27°C) between 21–23 min postspawning most consistently produced tetraploid embryos. Shocking the embryos at this time immediately precedes the disappearance of egg-jelly and the first division in mitosis, which begins at 30 min postspawning (Hudinaga 1941).

Shock duration was also shown to significantly affect induction rates, with a 10 min duration resulting in more tetraploid embryos compared with the 5 min duration. Li et al. (2003) also suggested that extended treatment durations may increase tetraploid induction rate. This likely results from shrimp spawning over a period of time, allowing more embryos to reach the embryonic developmental phase at which mitotic division can be prevented when a longer duration of shock is applied. Notably, no tetraploid *M. japonicus* embryos were produced at the longer 15 min duration. This is likely caused by the prolonged duration negatively interfering with the next stages of embryonic development.

Although tetraploid *M. japonicus* embryos were produced, they were nonviable, as determined by FACS analysis of the nauplii. Problems with tetraploid viability have been reported across a range of aquatic species. Li et al. (2003) reported similar problems for *F. chinensis* and were unable to produce viable tetraploid postlarvae by preventing the first division in mitosis. Qui et al. (1997) documented low viability among tetraploid freshwater prawns (*Macrobrachium nipponense*). Poor viability of tetraploids is commonly reported in numerous fish and shellfish species (e.g., Grass carp, *Ctenopharyngodon idella*, Cassani et al. 1990; Black carp, *Mylopharyngodon piceus*, Rothbard et al. 1997; Sydney rock oysters, *Saccostrea commercialis*, Nell et al. 1998; Manila clams, *Ruditapes philippinarum*, Diter & Dufy 1990; Pacific oysters, *Crassostrea gigas*, Guo 1991, cited by Guo & Allen 1994; Guo et al. 1994 and scallops, *Chlamys azumapekten*, Yang et al. 1997).

The genetic, biochemical or biological mechanisms that prevent tetraploid embryos from developing and hatching into viable nauplii remains unknown. In this study, it is unlikely that temperature shock alone was the cause of embryo deaths. This was evidenced by the presence of diploid nauplii hatching within the same temperature shock treatments as the nonviable tetraploids. One explanation proposed by Guo (1992) is that the diploid eggs have insufficient cytoplasmic reserves to develop as a tetraploid. Li et al. (2003) gives no explanation for their nonviable *F. chinensis* tetraploids, however, concluded that tetraploid embryos may have limited viability or ability to hatch.

In this study 6-dimethylaminopurine (6-DMAP) was also assessed as a potential shock agent to prevent the first division in mitosis. However, this treatment was not effective in inducing tetraploidy. Temperature and cytochalasin-B are the only shock agents that have been reported elsewhere to successfully inhibit the first division in mitosis in penaeid shrimp (Xiang et al. 1993, Li et al. 2003).

#### CONCLUSION

This study shows that the first division in mitosis can be inhibited in *Marsupenaeus japonicus* to produce tetraploid embryos. The most suitable treatment regimen for inducing tetraploidy in *M.*

*japonicus*, giving frequency and induction rate equal importance, was a 36 °C shock administered 23 min postspawning for a 5 or 10 min duration. Notably, none of the tetraploid embryos produced in this study were viable. If sterilization of penaeid shrimp is to be achieved through tetraploidy, future studies will first need to determine why tetraploid embryos are not viable.

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## PREPARATION OF LARGE BIVALVE SPECIMENS FOR SCANNING ELECTRON MICROSCOPY USING HEXAMETHYLDISILAZANE (HMDS)

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**ABSTRACT** Treatment with hexamethyldisilazane (HMDS) for drying specimens for Scanning Electron Microscopy (SEM) is described for relatively large (>20 mm shell height, SH) individuals of two scallop species, *Placopecten magellanicus* and *Argopecten irradians*. The traditionally used method of critical point drying (CPD) has been applied successfully for smaller specimens (~350 µm up to 15 mm SH); however, occasional rupture of the mantle occurred in these specimens. Furthermore, size limitation of the CPD chamber did not permit preparation of larger individuals. An alternative experimental protocol using HMDS was tested, which successfully preserves the morphology of the bivalve feeding organs, mantle surface and the cilia of the two scallop species with negligible shrinkage and few artifacts. Another benefit of this method is that the number of specimens and sample size is not restricted.

**KEY WORDS:** bivalve, scanning electron microscopy, method, hexamethyldisilazane (HMDS), ciliation, shrinkage

### INTRODUCTION

Scanning electron microscopy (SEM) is a very useful technique to study morphology and surface microstructure of various biological specimens in three dimensions. The drying process is one of the critical steps in specimen preparation for SEM (Laforsh & Tollrian 2000). However, removing water from specimens, particularly hydrated organisms normally causes distortion and collapse because of the effects of surface tension. The two principal methods used to reduce distortion during specimen drying for SEM are critical point drying (CPD) and freeze drying or lyophilization (Maugel et al. 1980, Boyde 1980). Air drying after preparation in organic compounds with low surface tension such as Peldri II (Brown 1990), hexamethyldisilazane (HMDS) (Nation 1983), tetramethylsilane (TMS) (Dey et al. 1989) and dimethoxypropane (DMP) (Muller & Jacks 1975) has also been used.

Critical point drying was first proposed by Anderson (1951) and is one of the techniques more commonly used to dry biological specimens for SEM (Maugel et al. 1980, Boyde 1980). Although we have used CPD for smaller bivalve specimens, namely sea scallop postlarvae, *Placopecten magellanicus*, ~350 µm to 15 mm in shell height (SH), with excellent results (Barré 2001, Veniot et al. 2003), intermittent rupture of the fragile mantle occurs with this method. Additionally, size limitations of the CPD chamber do not permit the use of this method for larger animals (>20 mm). In this study larger bivalve specimens were dried using a HMDS protocol that minimizes shrinkage and preserves the cilia and fragile mantle of the organisms.

The principle of the technique relies on replacement of an ethanol dehydration medium with HMDS, which is subsequently evaporated. HMDS has a lower surface tension than water and is believed to provide structural support by cross-linking proteins (Nation 1983, Braet et al. 1997). The suitability of HMDS for drying biological specimens has been shown on insects (Nation 1983, Heraty & Hawks 1998), aquatic organisms (shrimp eggs, daphnids, gastroticbs) (Moraes & Bouzon 1995, Hochberg & Litvaitis 2000, Laforsh & Tollrian 2000), organic microstructures on bivalve shells (Schöne & Bentley 2002), pollen (Chissoe et al. 1994), dinoflagellates (Botes et al. 2002), anaerobic biofilms and

granular sludge (Araujo et al. 2003), and cultured rat hepatic endothelial cells (Braet et al. 1997). Most of the studies have examined insects and small specimens, typically <10 mm.

Here we use SEM to show the morphology of feeding organs (gill, foot, labial palps and arborescent lips), and ultrastructural characteristics such as ciliation in large specimens of two bivalve species, the sea scallop, *Placopecten magellanicus*, and bay scallop, *Argopecten irradians*. An experimental protocol has been used that successfully preserves the organ morphology and cilia of large bivalves (>20 mm SH). Our observations will contribute to understanding the feeding mechanisms of these animals during development.

### MATERIALS AND METHODS

Large specimens (>20 mm SH) of the two scallop species *P. magellanicus* and *A. irradians* were collected for examination by SEM. Scallops were sampled from field sites and reared at the Institute for Marine Biosciences' Marine Research Station (MRS), Ketch Harbour, Nova Scotia, Canada.

Animals were refrigerated at 4°C for one hour prior to anesthetization to decrease their metabolism. A gradual addition of MS-222 (Tricaine Methanesulfonate) and KCl (5% potassium chloride) allowed for both muscle tissue relaxation (gaping valves and non contracted tissues) and limitation of mucus production. During this process, sample vials (250-mL glass jars) were not agitated and kept at 4°C. Scallops were fixed in 1G4F (1% glutaraldehyde, 4% formaldehyde, in 0.2M PO<sub>4</sub>, pH = 7.5) for a minimum of 48 h at 4°C.

During subsequent processing, samples were gently agitated in a fume hood at room temperature. Specimens were rinsed twice for one hour in a 0.2 M PO<sub>4</sub> buffer solution (pH = 7.2) and dehydrated in ascending ethanol washes (twice at each concentration for one hour in 50, 70, 80, 95 and finally 100% ethanol). Transitional steps of 100% ethanol (30 min, 30 min, 1h) and then HMDS: ethanol (1:1) (30 min, 30 min, 1 h) were followed by two baths (1 h each) of 100% HMDS. Finally the jars were placed in a dessicator with silica gel with just enough HMDS to cover the specimens.

The gross morphology of the scallops was observed using a Wild M400 Photomakroskop and a Nikon Coolpix 4500 digital

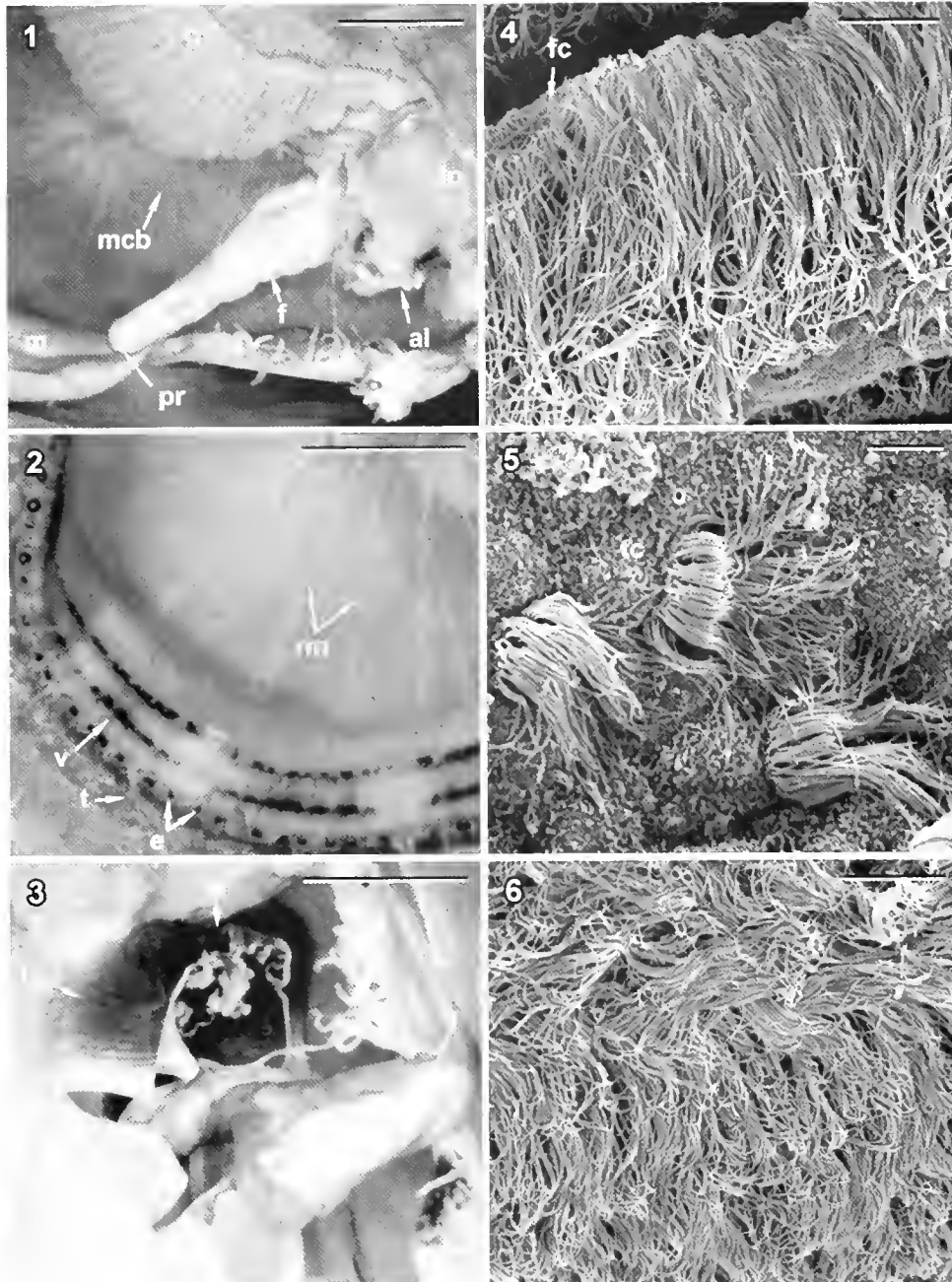


Plate 1: Results of HMDS treatment on the organization of the organs (binocular microscope observations) in the sea scallop, *Placopecten magellanicus* (Fig. 1, 2, 3) and on microstructure as examined by Scanning Electron Microscopy in the bay scallop, *Argopecten irradians* (Fig. 4, 5, 6). (See Veniot et al. 2003 for a description of the morphology and organization of feeding organs during ontogeny in *P. magellanicus*). Figure 1, General view of a specimen 22.4 mm in SH (*P. magellanicus*) showing the intact and relaxed feeding organs: arborescent lips (al), foot (f), gill (g), labial palps (lp), and associated mantle (m), and mantle ciliary band (mcb). Scale bar = 2 mm. Figure 2, Close-up of an intact mantle showing the eyes (e), the radial muscles on the mantle surface (rm), the sensory tentacles (t), and the velum (v). SH = 22.4 mm. Scale bar = 2 mm. Figure 3, Oral region showing the arborescent lips enclosed by the paired, ridged labial palps, the extended foot, and the connection between the gill and the labial palps for a sea scallop (*P. magellanicus*) 34.8 mm in SH. Scale bar = 2 mm. Figure 4, Frontal ciliation (fc) of an ordinary gill filament showing simple long cilia in *A. irradians* 21.5 mm in SH. Scale bar = 10  $\mu$ m. Figure 5, Tufts of cilia (tc) present on the dorsal expansions of the gill in *A. irradians* 21.5 mm in SH. Scale bar = 5  $\mu$ m. Figure 6, Ciliation of the ridge of the labial palps in *A. irradians* 21.5 mm in SH. Scale bar = 10  $\mu$ m.

camera. For SEM observation, specimens were mounted on aluminum stubs with double-sided carbon tape and the right valve was removed. Samples were then sputter-coated with gold-palladium (Model SC7620 Polaron) and examined using a Hitachi Model S-3000N SEM at 20kV.

## RESULTS AND DISCUSSION

Specimens of *P. magellanicus* dried after HMDS treatment showed well-preserved surfaces with negligible shrinkage of the tissues and few artifacts (Plate 1, Fig. 1, 2, 3). The extended foot

and intact gill (Plate 1) were relaxed and showed good preservation. The fragile mantle surface was well preserved with no shrinkage or tears (Plate 1, Fig. 2). The radial muscles were visible and well preserved. Feeding organs (lips, labial palps and foot) were intact and the arborescent lips (described and drawn by Beninger et al. 1990) were photographically recorded for the first time (Plate 1, Fig. 3). The labial palps–gill connection (type III according to Satsek 1963) was also shown (Plate 1, Fig. 3).

HMDS drying also provided excellent results in the preservation of microstructural characteristics such as ciliation of scallop feeding organs, as illustrated in the gills and labial palps of *A. irradians* specimens (Plate 1, Fig. 4, 5, 6). There were no visible distortions of the cilia as shown on the frontal cilia of the gill (Plate 1, Fig. 4) or the cilia of the ridges of labial palps (Plate 1, Fig. 6). Tufts of cilia on dorsal expansions of the gill were not shrunk and cilia at the base of the tufts were not clumped (Plate 1, Fig. 5).

HMDS treatment has been used for a number of different specimen types, generally less than 10 mm and was shown to require less time and expertise than critical point drying (Hochberg & Litvaitis 2000, Laforsch & Tollrian 2000, Schöne & Bentley 2002). The method is used in this study to prepare large specimens of scallops *P. magellanicus* and *A. irradians* for observation of feeding organs and cilia development. Oshel (1997) showed that HMDS use was effective for preparing soft and highly hydrated specimens. Also, HMDS drying preserved excellent surface details in insect tissues (Nation 1983, Heraty & Hawks 1998) and hepatic endothelial cells (Braet et al. 1997). This work shows that large specimens (20–30 mm SH) of marine bivalves can be preserved

and dried for SEM without major distortion or collapse and microstructural details are retained.

Intermittently, when using the CPD method for smaller specimens (scallop <15 mm SH) the mantle surface was partially or totally destroyed. All of the samples in this study consistently had intact mantle surfaces. Another difficulty encountered in the preparation of ciliated epithelia for SEM is the tendency for the cilia to stick together and for foreign particles to remain on the cilia. The ciliated surfaces of the feeding organs in *A. irradians* dried using HMDS showed nonclumped, relatively particle-free tufts (Plate 1, Fig. 4, 5, 6). Drying from HMDS seemed to prevent shrinkage, whereas the CPD method appeared to have caused some distortions (Barré 2001). Another advantage of the HMDS method is that the number of specimens and sample size are not restricted. Thus we conclude that the quality of the results obtained and ease of use of the HMDS drying process make it preferable to the CPD method for processing large bivalve specimens.

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## A NEW *IN SITU* METHOD FOR MEASURING SESTON UPTAKE BY SUSPENSION-FEEDING BIVALVE MOLLUSCS

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**ABSTRACT:** The most commonly used methods for measuring the amount of seston removed from the water column (uptake) by populations of suspension-feeding bivalve molluscs involve taking discrete water samples followed by laboratory analyses. Here we describe a new method based on *in situ* fluorometry that provides rapid measurement of seston removal rates. The new system is comprised of two identical units, each consisting of an *in situ* fluorometer, data logger and peristaltic pump with plastic tube attached to a deployment device. The deployment device allows precise placement of the fluorometer probe and intake end of the plastic tube so that *in situ* fluorescence (chlorophyll *a*) can be measured and water can be sampled for seston analyses in the laboratory from the same height. The typical setup involves placing one unit upstream and the other downstream of the study area and sampling the water at periodic intervals. Changes in seston concentration are revealed in the field by the fluorometers, and the sampled water can be analyzed in the laboratory for various seston parameters. Comparisons of the *in situ* data with data from laboratory analyses of pumped water samples were made for three species at four study sites: the eastern oyster (*Crassostrea virginica*), hard clam (*Mercuraria mercenaria*), and blue mussel (*Mytilus edulis*). Comparisons of measured upstream versus downstream seston concentrations indicated significant (*t*-tests,  $P < 0.05$ ) differences (uptake) for six of eight trials based on *in situ* fluorometry, but only marginally significant ( $P < 0.10$ ) differences at two of the four trials using laboratory chlorophyll *a* measurements. These data demonstrate that compared with sampling methods requiring laboratory analyses, the new *in situ* method provides much more rapid quantitative assessments and may provide more accurate estimates.

**KEY WORDS:** bivalve, fluorometry, seston uptake, suspension-feeding, *Crassostrea*, *Mercuraria*, *Mytilus*

### INTRODUCTION

Empirical and theoretical research demonstrates that populations of suspension-feeding bivalve molluscs can remove substantial amounts of suspended particulates (seston) from the overlying water column by their feeding activities (Ulanowicz & Tuttle 1992, Dame 1996, Newell et al. 2005, Haamer & Rodhe 2000, Cressman et al. 2003, Nelson et al. 2004). This has important implications for shellfish aquaculture and more recently for water quality management associated with shellfish restoration projects (Luckenbach et al. 1999, 2005, Thayer et al. 2005). Field studies on seston uptake typically involve laboratory analysis of discrete water samples obtained manually or by pumps (Dame & Libes 1993, Newell & Shumway 1993, Cressman et al. 2003, Nelson et al. 2004). This approach is effective and it allows the measurement of multiple water parameters. However, there is a need to develop *in situ* approaches that have the potential to greatly increase the spatial and temporal resolution of measurements of the feeding/seston uptake process.

A major impetus for development of the new method is the need for quantitative success metrics for constructed shellfish (mainly oysters) reefs that are part of ongoing restoration programs in many areas. These projects often emphasize the ecological functions of oysters, instead of or at least in addition to, their historical role as a commercial resource (Luckenbach et al. 1999, 2005, Brumbaugh et al. 2000, Coen & Luckenbach 2000). One of the major ecological functions of oyster reefs is their potential influence on water quality because of their filtration capacity (Dame 1996, Dame et al. 2001, French McCay 2003, Peterson et al.

2003). Field studies, however, typically have not demonstrated measurable seston removal by natural (Dame & Libes 1993, Wilson-Ormond et al. 1997) or restored (Nelson et al. 2004) oyster reefs. Cressman et al. (2003) is the only study we are aware of that measured substantial (up to 25%) removal of seston by small intertidal reefs. Hence, there is a need to critically assess this often-cited reason for restoring shellfish reefs (Coen & Luckenbach 2000, Luckenbach et al. 2005). How much of an impact on water quality should be expected from restored shellfish reefs?

Previous research has used *in situ* fluorometry to assess food availability to cultured bivalves (Grant & Bacher 1998) and phytoplankton biomass as part of broader ecological studies (Gregor et al. 2005). However, we are not aware of previous attempts to use *in situ* fluorometry to measure seston uptake by benthic organisms in the field. Here we describe a novel method for directly measuring uptake rates, and the results of field trials involving three bivalve species that compare measurements using the new *in situ* method with laboratory analysis of pumped water samples.

### DESCRIPTION OF *IN SITU* FLUOROMETRY APPARATUS

Each apparatus consists of a fluorometer (Seapoint Sensors Model SCF) with multimeter/datalogger (Extech Model 383.274), and peristaltic pump (Masterflex Model 7533) with 1-cm ID plastic tube attached to a custom-made deployment device (Fig. 1A, B, C). The fluorometer probe is placed within a 5-cm ID PVC pipe that is attached to the bottom plate. The probe and light shield are held in position by a hose clamp on the outside of the PVC pipe; loosening of the clamp allows the light shade and probe to be moved up and down on the pipe and secured at any height above the bottom plate. The bottom plate is designed to rest on the bottom so the fluorometer probe remains at the same height

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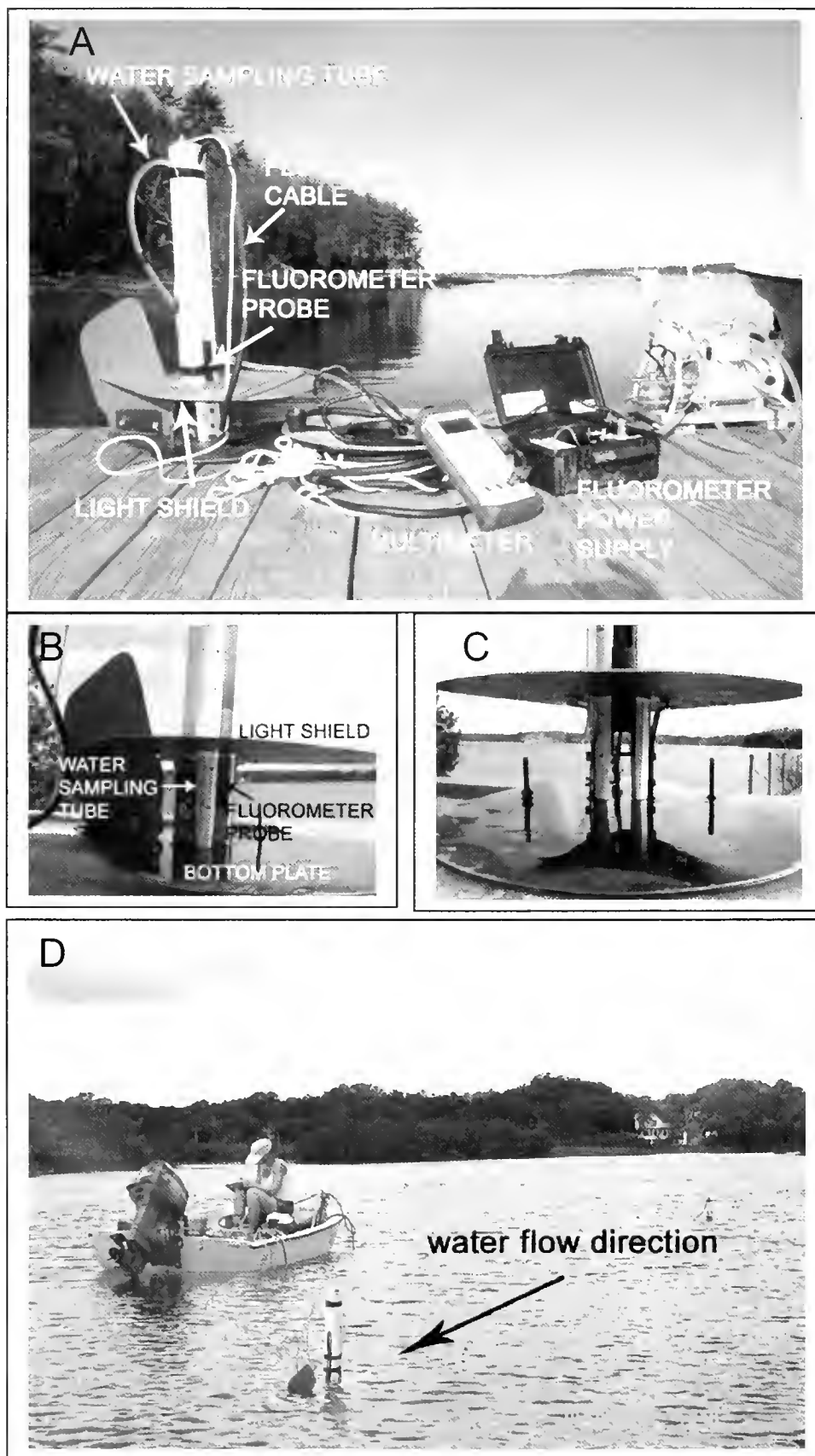


Figure 1. New *in situ* fluorometer and water sampling apparatus. (A) Complete apparatus (one of two identical units); (B) close up oblique view of fluorometer probe in deployment device; (C) close up face-on view; (D) deployment of two identical units over intertidal oyster reef in Florida.

throughout the measurement period, but the entire apparatus can be suspended at any height and moved up and down in the water column as needed. A tail fin attached to the bottom plate orients the probe so that water flows directly through the sensing chamber as it is lowered to the bottom (Fig. 1C). The bottom plate is constructed of 2-mm thick stainless steel sheet material and provides sufficient weight to hold the apparatus in position under most flow conditions. The fluorometer probe and the end of the water intake tube are placed at the same height so that comparisons can be made between *in situ* fluorescence (chlorophyll *a*) and laboratory analysis of pumped water. Total cost of each unit is about \$5,000 (2006 US\$).

The typical setup involves placing one apparatus upstream and another downstream of the study population and sampling the water at periodic intervals (Fig. 1D). To be sure the downstream apparatus is exposed to the same water mass as the upstream apparatus, a floating object is released periodically from the upstream position to show the ambient water flow path across the reef. Adjustments in location of either device, including raising the apparatus to allow the tail fin to reorient the probe with respect to water flow direction, can be made as needed. Changes in seston concentration caused by bivalve feeding ("seston uptake") are revealed immediately by differences in the two fluorometer readings (upstream vs. downstream), and the sampled water can be analyzed for various parameters to verify the fluorometry and provide additional data on changes in seston characteristics.

#### MATERIALS AND METHODS

Populations of bivalve molluscs were studied at sites in New Hampshire (a blue mussel, *Mytilus edulis*, reef), Virginia (hard clam, *Mercenaria mercenaria*, beds at an aquaculture farm), South Carolina (a restored eastern oyster, *Crassostrea virginica*, reef), and midAtlantic Florida (several natural eastern oyster reefs). Table 1 summarizes the environmental characteristics of the study sites, and the measured seston uptake rates. Sites were chosen because they met two criteria: (1) water less than ~1.5 m deep and (2) water flow constrained laterally, or width-to-length ratio sufficient to minimize lateral transport across the width of the sampled area. Meeting these criteria would likely result in environmental conditions (e.g., well mixed water column) that would allow sampling at one height to be representative of the entire flow field.

The general protocol for field studies consisted of making repeated measurements of environmental conditions and changes in seston concentration upstream and downstream of each population of bivalves based on *in situ* fluorometry and laboratory analysis of pumped water samples (Table 1; Fig. 1C). Each of the fluorometer probes and water intake tubes were set at 5–10 cm above the bottom (either the top of the reef for mussels and oysters or the sediment surface for clams). A set of measurements (each consisting of 10–20 fluorometry readings recorded at 10-s intervals) was made at 10-min intervals for the duration of each deployment at each site (except for the South Carolina study, which consisted of single readings recorded at 5–15-min intervals). The peristaltic pumps required about 5 min to obtain each sample. Readings from the two fluorometers also were compared side-by-side at the beginning of each deployment and again after the last set of readings was taken to be sure they gave similar readings.

Pumped water samples were stored in the dark on ice and returned to the laboratory for filtration (Whatman GF/C or Gelman GF/F filters) within 6 h of collection; the filters were further pro-

TABLE 1.  
Summary of environmental characteristics, bivalve population data, and other information for all study sites.

Species	Bivalve Density (#/m <sup>2</sup> )	Mean Shell Size (mm)	Location	Site	Date	Tide	Sampling Duration (hr)	# of Samples		Water Depth Range (m)	Flow Length (m)	Flow Speed Range (cm/s)	<i>In situ</i> Fluoro (% uptake)	Laboratory Chl <i>a</i> (% uptake)
								<i>In situ</i> Fluoro	Pumped Water					
<i>Mytilus edulis</i>	526	45.0	New Hampshire	Albacore Channel	11/13/01	Ebb	1.4	4	4	0.32–0.47	63	12.6–27.8	27.8	11.8
<i>Mercenaria mercenaria</i>	285	19.4	Virginia	Clam Bed 1	6/5/02	Flood	0.8	5	4	0.35–0.42	9	10.0–13.0	35.3	16.1
<i>Mercenaria mercenaria</i>	285	19.4	Virginia	Clam Bed 2	6/6/02	Ebb	0.4	3	0	0.61–0.65	44	3.0–5.0	62.3	
<i>Crassostrea virginica</i>	61	36.8	Florida	CANA Reef 1	6/10/02	Flood	1.0	7	0	0.28–0.34	12	12.0–17.0	11.4	
<i>Crassostrea virginica</i>	122	54.9	Florida	CANA Reef 2	6/10/02	Ebb	0.7	4	0	0.18–0.19	20	3.0–4.0	37.4	
<i>Crassostrea virginica</i>	76	50.5	Florida	CANA Reef 3	6/11/02	Ebb	1.8	8	6	0.40–0.50	20	4.0–6.0	10.7	11.9
<i>Crassostrea virginica</i>	134	47.0	Florida	CANA Reef 4	6/12/02	Flood	1.3	4	0	0.17–0.18	17	8.0	26.3	
<i>Crassostrea virginica</i>	2538	26.7	South Carolina	Palmetto Reef 1	10/18/04	Flood	1.7	11	3	1.0–1.5	6	3.5–11.5	–2.7	–5.3

cessed immediately in most cases, or frozen until processed. The New Hampshire, Florida and Virginia samples were analyzed spectrophotometrically using acetone extraction and standard techniques (APHA 1992). The South Carolina samples were analyzed fluorometrically following a modified EPA Method 445.0 procedure (Arar 1997, Rev. 1.2).

Bivalve densities were determined at most sites by sampling 5 to 10 of 0.16 m<sup>2</sup> quadrats, counting and measuring shell length or height of all live bivalves collected to the nearest mm using calipers. For the South Carolina oyster reef, data on bivalve size and density available from previous samples taken at that site were used. Flow length was the distance between the two fluorometers. Water depth was measured at 10-min intervals using a marked rod. Water flow speed was measured at the same height as the fluorometer probes were placed (5–10 cm above the bottom) with a Marsh-McBirney Model 201 electromagnetic current meter, with 10–20 replicate speeds recorded at 10-s intervals every 10 min for the duration of the measurement period. Near-surface water speed and flow direction were also estimated by releasing an orange at the upstream site and recording travel time to the downstream site. This provided a measurement of near-surface water flow speed to compare with the near-bottom measurements and insured that the sampling units were placed properly.

Data analysis consisted of comparisons of chlorophyll *a* concentrations, based on field data (*in situ* fluorometry) with laboratory analysis of pumped water samples. Data were analyzed graphically (scatterplots), and statistically (*t*-tests, regression and correlation) using SYSTAT version 10 (2000) software. For the *in situ* fluorometry data only, the *t*-tests were done using the means of the mean values for each 10-min observation period.

## RESULTS AND DISCUSSION

A total of eight trials of paired upstream versus downstream *in situ* fluorometry measurements were taken at the four study areas; pumped water samples were taken for laboratory chlorophyll *a* analysis during four of the eight trials (Table 1). *T*-tests indicated significant differences between mean upstream and downstream fluorometry readings for six of the eight trials (Fig. 2), but only marginally significant ( $P < 0.10$ ) differences at two of the four trials for laboratory measurements (Fig. 3). Both approaches, however, showed instances of seston uptake rates exceeding 25%.

A scatterplot of the full dataset comparing each *in situ* fluorometry reading with its corresponding laboratory chlorophyll measurement showed two distinct groupings (Fig. 4). Overall, this assessment indicated that the two measurement techniques yield comparable data, but their relationship is not simple.

### Using *In situ* Fluorometry to Provide Rapid Measurement of Seston Uptake

As already noted, field studies on seston uptake typically have involved laboratory analysis of discrete water samples obtained manually or by pumps using various sampling protocols (e.g., Dame & Libes 1993, Judge et al. 1993, Newell & Shumway 1993, Cressman et al. 2003, Nelson et al. 2004). We obtained pumped water samples for laboratory analysis as means of "ground truthing" the *in situ* fluorometry data because in the long-term our goal is to rely as much as possible on the latter. Most applications of *in situ* fluorometry to date have been in the area of water quality monitoring (e.g., Gregor et al. 2005), in some cases (e.g., Grant & Bacher 1998) related to bivalve aquaculture. Our application es-

entially represents an ecological extension of the method, but it required some important modifications to off-the-shelf fluorometers. Ambient sunlight can strongly interfere with the sensor, so a large aluminum plate was added to shade the probe (Fig. 1). It was also necessary to construct a deployment apparatus that allowed precise placement of the probe vertically, and it automatically oriented the probe so the predominant water flow was directly through the sensor chamber. These modifications have resulted in an apparatus that is simple to deploy and appears to consistently yield reliable data.

*In situ* fluorometry could become a fast, effective and nondestructive approach to quantifying the impacts of shellfish populations on seston removal but it needs further development, including more comparisons with laboratory analysis of pumped water samples. One of the issues that will need to be settled for some applications concerns the relationship between the *in situ* fluorometry data and laboratory-determined chlorophyll *a* concentrations. Our data from four different areas showed two distinct groups, with substantial disparity between the two in the relationship between fluorescence and chlorophyll *a* concentrations determined by wet chemistry techniques (Fig. 4). Grant and Bacher (1998) also reported substantial disparities between the two approaches. Gregor et al. (2005) noted that differences between *in situ* fluorometric methods and laboratory methods should be expected when different analytical techniques (e.g., ethanol vs. acetone extraction) are compared and phytoplankton taxonomic composition varies. For our data, the pumped water samples were a composite taken over about 5 min, as compared with multiple fluorometry reading taken over about 2 min (see Methods section). Hence, although both types of samples were taken at approximately the same time, some of the differences (Fig. 4) could represent actual temporal variability in seston concentrations.

Another potential limitation of *in situ* fluorometry is that it does not provide data on components of the seston other than phytoplankton that can be important food items for bivalves. However, if chlorophyll *a* data alone are sufficient then *in situ* fluorometry represents a much more effective approach compared with analysis of pumped water samples because it provides rapid results, and if fluorometers are deployed at multiple heights in the water column, spatial variations in seston removal that are related to hydrodynamical factors can be assessed. For example, a fully mixed water column is not necessary for estimating uptake rates.

### Seston Uptake by Oyster Reefs

As discussed earlier, the need to estimate the potential impact of constructed or restored oyster reefs on water quality has driven the development of the *in situ* fluorometers. The potential for bivalve shellfish to control phytoplankton populations in coastal areas such as San Francisco Bay was proposed over 20 y ago (Cloern 1982, Officer et al. 1982). Subsequent studies in the Bay documented a variety of ecological effects, including seston depletion, attributable to dense infaunal bivalve populations (e.g., Alpine & Cloern 1992). In a widely cited paper, Newell (1988) hypothesized that the historical depletion of oyster populations in Chesapeake Bay has been a major factor in water quality degradations and other ecological changes in the Bay (also see Heck 1987). Subsequent studies support this notion (Ulanowicz & Tuttle 1992, Newell 2004). Similar water quality impacts in some areas of the Great Lakes by the invasive zebra mussel have also been documented (Budd et al. 2001, Ackerman et al. 2001). Recent research in mesocosms has further characterized the role that sus-

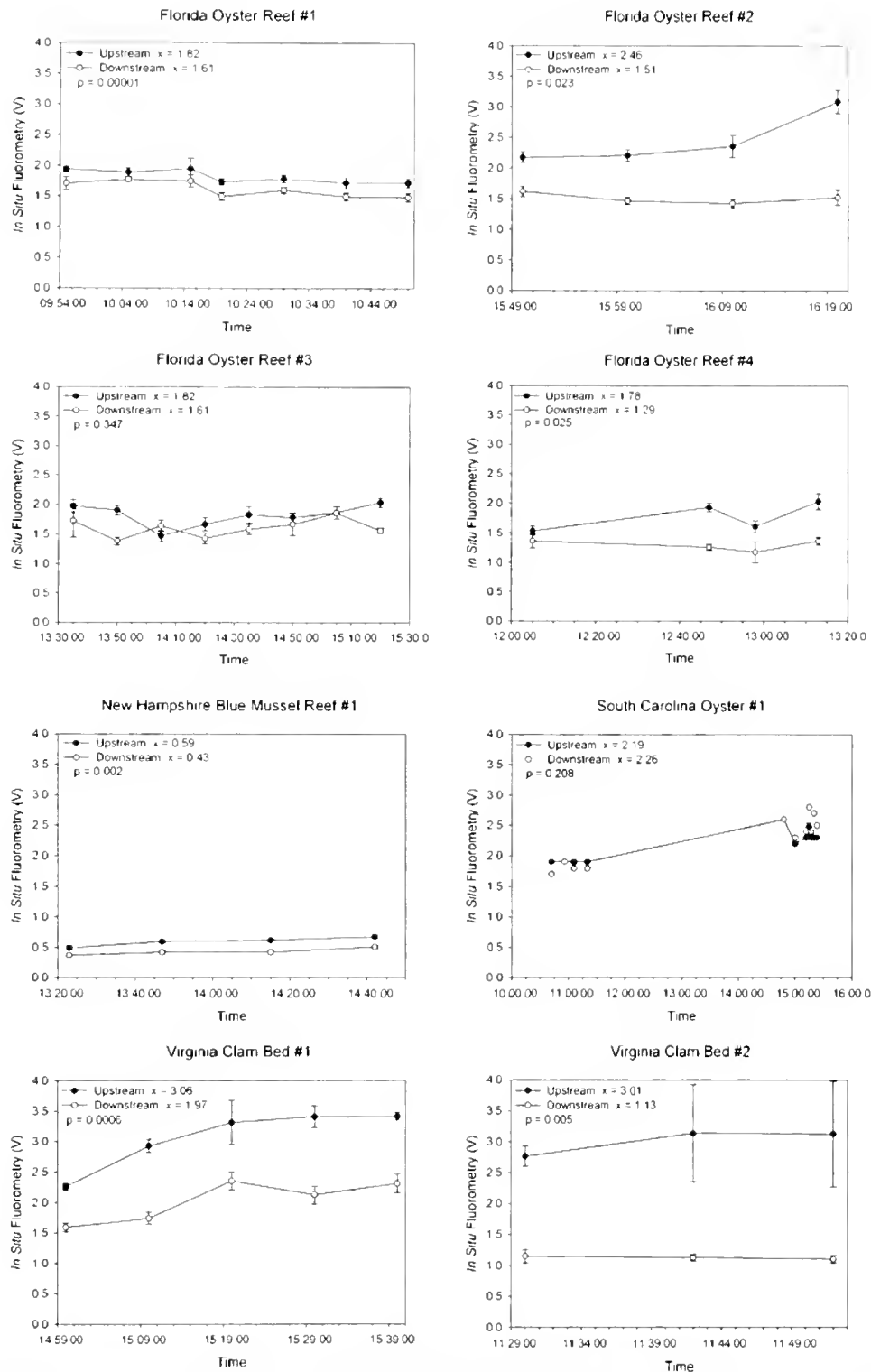


Figure 2. Upstream and downstream *in situ* fluorometry measurements for all study sites. *P* values from *t*-tests; error bars show 1 SD.

pension-feeding bivalves can play in controlling phytoplankton populations (Cerrato et al. 2004, Porter et al. 2004). It seems reasonable to expect measurable water quality effects from restored oyster reefs, but empirical studies of the effects are needed.

Several field studies involving bivalves such as clams, mussels and other taxa have demonstrated substantial seston uptake and in

some cases longer-term water quality changes caused by bivalve feeding and filtration (e.g., Alpine & Cloern 1992, Haamer 1996, Coen et al. 2000, Haamer & Rodhe 2000, Ackerman et al. 2001; see reviews by Dame 1996 and Dame et al. 2001). Field studies on oysters, however, typically have shown no measurable uptake or very little (Dame & Libes 1993, Wilson-Ormond et al. 1997, Nel-

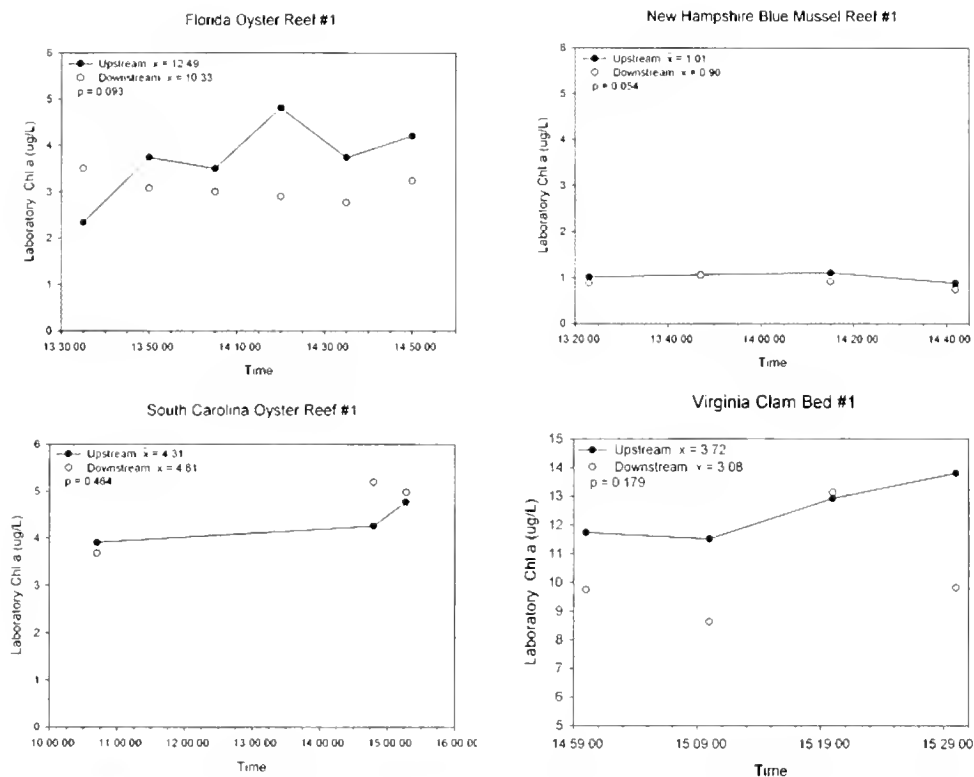


Figure 3. Upstream and downstream laboratory measurements of chlorophyll *a* concentrations from pumped water samples for all study sites. *P* values from *t*-tests.

son et al. 2004); Cressman et al. (2003) is the only field demonstration of substantial seston removal (up to 25% decreases in chlorophyll *a*) we are aware of for oyster reefs. In the present study, maximum measured seston uptake rates for the three bivalve species ranged from 27.8% for *Mytilus*, 37.4% for *Crassostrea*, to 62.3% for *Mercentaria*. The differences in rates mainly reflected differences in bivalve size and densities relative to water flow and

water depth (Table 1). Hence, it seems reasonable to conclude that field studies on oysters should consistently yield quantitative measures of seston depletion if they are properly scaled. In any case, our new *in situ* device would allow rapid assessments for future studies on restored and natural oyster reefs.

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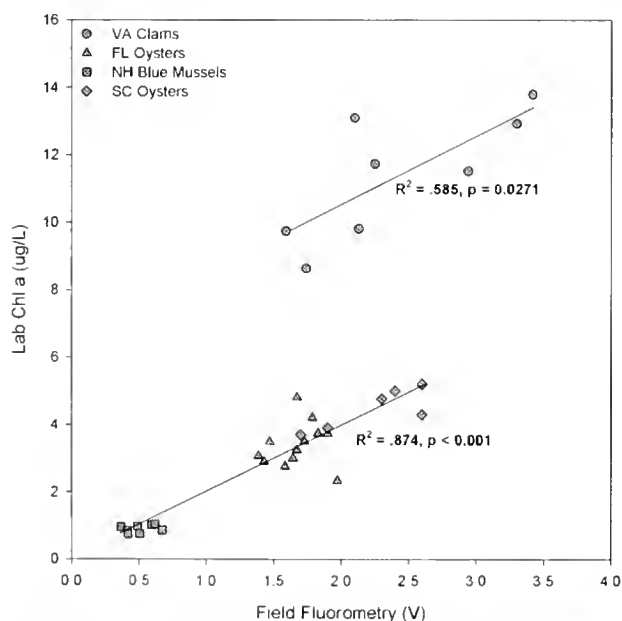


Figure 4. *In situ* fluorimetry compared with laboratory analysis of pumped water samples for all study sites.

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## **ABSTRACTS OF TECHNICAL PAPERS**

*Presented at the 26th Annual*

## **MILFORD AQUACULTURE SEMINAR**

Milford, Connecticut

February 27–March 1, 2006



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February 27–March 1, 2006

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**OVERVIEW, 26th MILFORD AQUACULTURE SEMINAR.**

**Walter J. Blogoslawski**, U.S. Department of Commerce, National Oceanic & Atmospheric Administration, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, 212 Rogers Ave., Milford, CT 06460, USA

In early 1975, fifteen persons gathered at the First Milford Aquaculture Seminar to discuss the role of industry, research, and government in managing the complex interactions of society's needs and the environment to shape the fledgling United States efforts in aquaculture. Twenty-six years later, one hundred and fifty-five attendees of the joint 26th Milford Aquaculture Seminar and the Juvenile Oyster Disease Session met from February 27–March 1, 2006 in Meriden, Connecticut to examine how well this important and difficult charge has been addressed and to discuss future challenges and opportunities in this evolving field.

This annual meeting is a unique blend of industry, academics, government, and research that results in sessions that are informative and in observations that prove invaluable because of the many ways an issue is examined depending upon the disciplines of the persons involved in the discussion. This collaborative approach brings the best of all worlds to topics as varied as disease, regulation, genetics and nutrition. Input is received from those who work primarily in a laboratory or a classroom and from those who spend their time in the hands-on process of animal husbandry, each receiving valuable insights from the other's experience. This easy partnering of experience and formal research helped the meeting to achieve the goal of sharing knowledge to achieve excellence in aquaculture practices consonant with a keen awareness of our stewardship of the environment.

The 26th Milford Aquaculture Seminar attracted 17 posters and 52 oral presentations on subjects covering disease, genetics, animal husbandry, shellfish restoration, aquaculture legislation, nutrition, technology, and an update on the NOAA Aquaculture Program. A concurrent Juvenile Oyster Disease (JOD) session explored industry cases, transmission, and treatment and resistance to JOD.

Participants came from thirteen US states and from the countries of Pakistan, Korea, France and Canada. Nineteen universities sent professors or students and two Connecticut magnet schools and one Massachusetts High School participated. Four universities sent NOAA-Sea Grant cooperative extension agents. Sixteen aquaculture companies attended as did several independent shellfish growers. Many marine laboratories participated including federal US laboratories from Milford, CT, Woods Hole MA, USGS Kearneysville, WV, NOS Hollings Marine Lab, Charleston, SC, USDA-NRAC, College Park and NOS Oxford, MD. Federal program information was brought to the meeting from the NOAA Aquaculture Program, Silver Springs, MD and NOAA Chesapeake Bay Office, Annapolis, MD. State institutes and municipal departments attending included the University of Maryland's Center for Marine Biotechnology, the Rhode Island Department of Environmental Management, New Hampshire Fish and Game, Oak Bluff's

and Edgerton, MA Shellfish Departments, the State of Connecticut Aquaculture Division, and the Connecticut Department of Environmental Protection.

The diversity of the participants, coupled with the high caliber of the presentations in an atmosphere that encouraged positive formal and informal discussion, ensured a dynamic and worthwhile meeting for all. Surely, the 26th Milford Aquaculture Seminar and the joint Juvenile Oyster Disease session addressed the need for such an information exchange and have remained faithful to the original intent of the First Milford Aquaculture Seminar—to make available the latest research and industry experience so that aquaculture might be conducted in a responsible and productive manner.

**PROTOCOL VALIDATION FOR THE USE OF THE FLUORESCENT ENZYME PROBE ELF97 TO ASSESS PHOSPHATE NUTRIENT STATUS OF INDIVIDUAL PHYTOPLANKTON CELLS.** Jennifer H. Alix,<sup>1</sup> Shannon L. Meseck,<sup>1</sup> Sebastian Saliou,<sup>2</sup> J. Evan Ward,<sup>3</sup> and Gary H. Wikfors.<sup>1</sup> <sup>1</sup>USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460; <sup>2</sup>Pâtisseries Gourmandes SA, 22602 Loudeac Cedex, France; <sup>3</sup>Department of Marine Sciences, UCONN, Groton, CT 06430

Methods used in the past to assess the nutrient status of phytoplankton populations have been indirect, inferred from changes in growth correlated with external, dissolved nutrient concentrations. A much more direct approach is to measure enzymes induced by nutrient uptake or deficiency within the phytoplankton cells themselves, but early methods required extraction of enzymes from bulk samples comprised of many cells, thus limiting resolution to the entire phytoplankton community in field samples or an average of all cells in a culture. Recently, fluorescent probes that bind uniquely to specific enzymes within cells have been developed, mainly for biomedical applications. We currently are testing cross-field application of several of these probes for use with phytoplankton cells. As phosphate status of phytoplankton is an important consideration in both natural and culture assessments of phytoplankton, we have focused on a probe, ELF97, for the phosphate reductase enzyme induced under conditions of phosphate deficiency. The general approach was to induce phosphate deficiency in phytoplankton cultures and compare fluorescence intensities of P-deficient and P-sufficient cultures; a significant difference indicates the ability to distinguish the two nutrient-status conditions.

Preliminary experiments were done to modify and optimize the details of sample handling and probe concentration from medical samples to phytoplankton. Then, 15 strains representing 10 different classes of algae were inoculated into artificial seawater medium, ASP<sub>2</sub>, with normal or zero phosphate enrichments. After two serial subcultures, dissolved phosphate concentrations and

particulate phosphorus were determined to ascertain the availability of this nutrient in the cultures. Probe fluorescence within cells was observed qualitatively by epifluorescence microscopy, and fluorescence intensity of individual cells was determined by flow-cytometry. In all algal species we were able to starve for phosphate, there was a significant increase in cellular mean probe fluorescence from P-sufficient to P-deficient nutrient status, indicating that this probe is widely-applicable for determining phosphate status of many phytoplankton taxa. Probes to test phytoplankton nitrate reductase, lipid content, viability, and apoptosis will be tested in the future

**VARIATIONS IN THE TIMING OF SPAWNING IN THE POPULATION OF WILD BAY SCALLOPS, *ARGOPECTEN IRRADIANS*, IN NANTUCKET HARBOR.** Peter B. Boyce, Robert S. Kennedy, Valerie A. Hall and W. Forrest Kennedy, Maria Mitchell Association, 4 Vestal Street, Nantucket, MA 02554, USA

During the summer and early fall of 2004 and 2005 we measured the timing of the spawning and subsequent set of the bay scallop *Argopecten irradians* population in Nantucket Harbor using the wet Gonad Index (GI) of samples collected at 2 to 3 week intervals and spat collecting bags set out at 3–6 week intervals. After eight weeks, or longer, the bags were collected and the number of small seed scallops in each bag were counted and measured if large enough to do so.

In 2004, the spawn began as a dribble in July with the main event occurring from mid-August to early September. Spat lines set early in the summer contained no more than a handful of seed, while a spat line deployed on 14 September 2004 and examined on 17 November averaged 350 small seed per bag with a mean size of  $3.3 \pm 1.2$  mm. The size distribution of the overwintering population was derived from a composite of all spat bag scallops plus samples obtained during 25 m<sup>2</sup> bottom transects. The predominant component was a subpopulation of small seed with a mean shell height of  $11 \pm 3$  mm. In addition there were a small number of larger size seed. No seed scallops with a shell height >40 mm were found.

The 2005 spat bag data indicate the main spawning event occurred in July with a significant dribble spawn occurring throughout August and into September, virtually a mirror image of the 2004 behavior. Spat lines with five bags set out at two places in the harbor on 24 July 2005 and checked on 28 September 2005 yielded an average of  $2,300 \pm 700$  and  $2,100 \pm 300$  seed per spat bag. Most of the overwintering population in 2005–2006 apparently originated from this spawning event. The amount of seed is larger than in the previous years and the individuals are also larger, with a mean shell height of  $52 \pm 4$  mm. This was 41 mm larger than the 2004 overwintering seed, and 10 mm larger than in 2003 when the mean shell height was  $42 \pm 4$  mm.

The GI was determined for samples of randomly selected adult

scallops during the 2004 and 2005 growing season. In 2004, the Gonad Index remained above 20 (indicating a full gonad) through the end of July before dropping to 7 (empty gonad) by September 15. In 2005 the onset of the GI decline was less well defined, beginning approximately June 19 and reaching low values before August 11.

Although the small seed from 2004 did survive the winter, it did not reach adult size in 2005. More than 98 percent of the 2005 population that we sampled consisted of adult scallops that had survived a second winter, exhibiting two annual growth rings, one at 40mm and the second at 50–65mm. The GI data for 2005 comes almost exclusively from these third season scallops.

**IN VITRO INVESTIGATIONS OF QUAHOG PARASITE UNKNOWN (QPX).** Deenie M. Buggé, Mickael Perrigault and Bassem Allam, Marine Sciences Research Center, Stony Brook University, Stony Brook NY 11794

Quahog parasite unknown (QPX) has been successfully isolated from clams from different geographic locations in the Northeast. This study uses an *in vitro* approach to investigate the virulence and specificity of various QPX isolates. Interactions between QPX and components from different bivalve species and strains were investigated. Our experiments indicate that QPX growth in *M. mercenaria* is tissue-specific and that some clam tissues possess anti-QPX properties. For example, host foot tissue supports *in vitro* QPX growth while growth in mantle or gill tissue is strongly inhibited. Bivalve plasma also contains anti-QPX factors which may play a role in host defense. Plasma from species not known to develop QPX disease contains higher anti-QPX activity than *M. mercenaria* plasma. Our results also demonstrate that QPX produces extracellular virulence factors that are cytotoxic to *M. mercenaria* hemocytes. This cytotoxicity may play an important role in supporting QPX infection and proliferation within the host. These and future *in vitro* investigations will increase our understanding of QPX infection and disease development. For instance, studies targeting tissues where anti-QPX activities are localized may provide a good strategy for the determination of biological bases of QPX resistance in clams.

**NOAA AQUACULTURE PROGRAM UPDATE.** Susan M. Bunsick, NOAA Aquaculture Program, 1315 East-West Highway, Silver Spring, MD 20910

As a Federal agency under the U.S. Department of Commerce, the National Oceanic and Atmospheric Administration (NOAA) is focused on creating domestic seafood supply to meet the growing demand for all seafood products. Currently, over 70% of the seafood Americans consume is imported, and at least 40% of those imports are farmed seafood. Domestic aquaculture can be an effective option to reduce dependence on seafood imports, provide jobs for economically depressed coastal communities, and increase



regional food supply and security. For years, NOAA and its partners have worked with coastal communities interested in exploring shellfish and finfish aquaculture as another method to produce seafood, provide jobs and revenues, and use existing seafood processing facilities. As it develops, offshore aquaculture will be one component of the broad NOAA aquaculture program, which currently addresses coastal and onshore marine shellfish and finfish farming. NOAA's Aquaculture Program also includes stock enhancement research and hatchery activities which support commercial and recreational fishing, endangered species and habitat restoration.

**DETECTION AND QUANTIFICATION OF NITRATE REDUCTASE IN THE DINOFLAGELLATE, *PROROCENTRUM MINIMUM*.** Shanna S. Chambliss and Gulnihal Ozbay, Delaware State University, Department of Agriculture & Natural Resources, 1200 North Dupont Highway, Dover, DE 19901; Gary H. Wikfors, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460; and Allen Place, Center of Marine Biotechnology, University of Maryland, Suite 236 Columbus Center, 701 E. Pratt Street, Baltimore, MD 21230

In marine environments, nitrate ( $\text{NO}_3^-$ ) is the major form of inorganic nitrogen used to support primary production. Nitrate reduction to nitrite ( $\text{NO}_2^-$ ) is attributed to nitrate reductase activity. The enzyme nitrate reductase is abundant if phytoplankton are using nitrate to promote growth, but it has been difficult to attribute nitrate reductase activity to specific organisms or groups of phytoplankton.

A new nitrate reductase substrate, 6-chloro-9-nitro-5-oxo-5H-benzof[a]phenoxazine (CNOB), shows promise in this regard. When a nitrate group is cleaved from the CNOB reagent, the remaining molecule precipitates near the site of enzyme activity, thus fluorescently tagging cells with nitrate reductase activity. We quantified CNOB labeling in axenic cultures of dinoflagellate, *Prorocentrum minimum*, to understand how CNOB labeling responds when nitrate levels vary. Axenic batch cultures of *Prorocentrum minimum* were grown in two different media types: medium type one with nitrate, using potassium nitrate ( $\text{KNO}_3$ ) as an inorganic nitrate source and, medium type two with  $\frac{1}{4}$  the  $\text{KNO}_3$  of standard medium.

Cultures were incubated over a period of 15 days at 20°C on a 12:12 light/dark cycle. On day 15 of the experiment, flow cytometry was used to detect and quantify the amount of CNOB retained in individual cells. Nitrate reductase, as detected by CNOB labeling, is present in cells during the late-log or early-stationary phase. Preliminary results indicate that cells grown in high-nitrate media show higher CNOB fluorescence activity when compared to cells grown in low-nitrate media. Our results indicate that CNOB is an excellent tool for detecting and quantifying nitrate reductase activity in the dinoflagellate, *Prorocentrum minimum*. By using flow

cytometry and CNOB to detect and quantify nitrate reductase activity in this study, we will gain a better understanding of the nutrient status of marine phytoplankton at the cellular level.

**COMPARISON OF GROWTH PERFORMANCE OF BAY SCALLOPS (*ARGOPECTEN IRRADIANS*) WITH PHENOTYPES OF DIFFERENT SHELL COLORS.** Joseph Choramanski, Sheila Stiles, Dorothy Jeffress and Dionna Williams, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460

Juvenile bay scallops with phenotypes of different shell colors and patterns from the same genetic line were compared for growth and survival. The phenotypes were divided into three groups: white, striped and the gray/brown typical of bay scallop coloration. Fifteen scallops were placed in 4.0 L beakers with three replicates for each shell color. Groups were further broken into three size classes: 5–7, 8–10 and 11–15 mm. Scallops were held in unfiltered sea water static cultures with aeration at ambient temperature. Beakers were fed daily with an algal mixture of *Tetraselmis* and *Rhodomonas*. Seawater was changed daily and scallops were measured weekly for six weeks. Final mean sizes for all three groups showed good growth, comparable to scallops in running seawater controls. Survival for all groups was above 80% and allowed for adequate sample sizes throughout the experiment. Based on ANOVA, there was no significant difference among the three groups for either growth or survival. This indicates that shell color in general for scallops from the same line is not a significant factor affecting these attributes. These findings also confirmed our previous results from studies that showed no significant difference among bay scallops with various shell phenotypes for growth and survival, including overwintering survival.

**IMMUNOMODULATION IN EASTERN OYSTERS, *CRASSOSTREA VIRGINICA*, EXPOSED TO A PAH-CONTAMINATED BENTHIC DIATOM.** April N. Croxton<sup>1,2</sup>, Gary H. Wikfors<sup>1</sup> and Richard D. Gragg, III.<sup>2</sup> <sup>1</sup>USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460; <sup>2</sup>Environmental Sciences Institute, Florida A&M University, Tallahassee, FL 32307

Benthic microalgae inhabiting surface sediments play an important role in shellfish-harvesting waters. These roles include sediment stabilization, primary production, and provision of a food source for bivalve species. As a source of nutrition for bivalves, benthic algae are also capable of serving as vectors transferring contaminants from sediments to bivalves. Hydrophobic contaminants can become adsorbed to benthic microalgae in the sediment, and subsequently resuspended microalgae may be ingested by suspension-feeders. This trophic transfer of toxic contaminants can

affect the physiology of a bivalve, including immune defense capabilities.

In this experiment, the effects of PAHs on the immune functions of the eastern oyster, *Crassostrea virginica*, were examined through a microalgal trophic pathway. Oysters were exposed to PAH-contaminated benthic diatoms to examine the effects of this contaminant on oyster immune defense functions. Hemocyte immune-defense functions, including adhesion, oxidative burst, phagocytosis, and viability, were measured using flow-cytometric methods. Significant changes in cellular immune status were associated with PAH exposure. Subsequently, eastern oysters collected from field sites suspected of being contaminated with PAHs will be analyzed to identify correlations in immune responses of these oysters with those exposed experimentally to PAHs in the laboratory. The goal of this research is to employ an ecosystem-based approach to studying the impacts of contaminants associated with a microalgal food source upon a harvestable bivalve species.

**PECONIC ESTUARY RESTORATION: A SPAT (SOUTHOLD PROJECT FOR AQUACULTURE TRAINING) ASSISTED PROJECT TO RECLAIM A LOST SCALLOP POPULATION.** Armand DeLuca, Otto Schmid, and Kim Tetrault. Cornell Cooperative Extension of Suffolk County Marine Program, Marine Environmental Learning Center, Southold, NY 11971

This presentation described the role of a mature, volunteer community-based, restoration group, *Southold Project for Aquaculture Training (SPAT)*, within a major shellfish restoration project.

This project was initiated in 2004 under a grant by the legislature of Suffolk County on Long Island, NY. The funding, almost \$1.8 M, was designated to support a restoration project with the goal of infusing 50 million scallops into the Peconic Bay Estuary over a period of four years.

As a result of a disastrous brown tide bloom that began in 1985 and occurred sporadically over the following decade, the once prolific scallop population in the Peconics has been reduced to a fraction of its original size. Restoration efforts to date have had only limited success. The theory exists, however, that a much larger reseeding effort might well encourage and jump-start an even-larger natural set. This, then, provided the impetus for the ambitious restoration project to which Cornell Cooperative Extension of Suffolk County (CCE) and Long Island University, NY, committed their efforts.

CCE's responsibility to this project is to provide the seed scallops for grow-out both in containment and for controlled bottom planting in designated sanctuaries in Peconic Bay. This effort includes hatchery and nursery operations and the ongoing maintenance of the juvenile scallops while in containment in open waters.

Long Island University is responsible for technical monitoring and encoding, including genetics and morphometrics, water quality and survival data.

This project has required far more physical resources than previously existed. Cornell Cooperative Extension, with the SPAT volunteers providing much of the labor by logging in 11,437 hours in 2005, has furnished the infrastructure needed to meet the requirements of the project. Over the past year, SPAT volunteers, working with CCE staff, have built a new nursery with raceway tanks, built a new algae room and installed a continuous SEACAPS algae system, constructed a large, fully-equipped 36 foot work barge for field maintenance, and a workshop for various support projects. These efforts have saved CCE hundreds of thousands of dollars by eliminating the need for outside contractors. The volunteers also work with CCE staff in maintaining the shellfish in various forms of containment from the larval stage, through post-set and grow-out, participated in a fall deployment of stock to sanctuaries and in preparing approximately 300,000 scallops for over-wintering in lantern nets for a spring deployment in 2006. The goal of the project is to produce upwards of 10–15 million post-set scallops per year.

**ASSESSING DISEASE TOLERANCE IN THE EASTERN OYSTER USING GENE EXPRESSION PROFILING.** Elie Diner,<sup>1</sup> Roxanna Smolowitz,<sup>1</sup> Marta Gomez-Chiarri,<sup>2</sup> Karin A. Tammi,<sup>3</sup> Dale Leavitt<sup>3</sup> and Steven Roberts.<sup>1</sup> <sup>1</sup>Marine Biological Laboratory, 7 MBL Street, Woods Hole, MA 02543; <sup>2</sup>University of Rhode Island, 20A Woodward Hall, Kingstown, RI 02881; <sup>3</sup>Roger Williams University, One Old Ferry Road, Bristol, RI 02809

Oyster populations along the east coast have been devastated by diseases such as Dermo, caused by the parasite *Perkinsus marinus*. Previous research has demonstrated that genetic factors can be selected that contribute to disease resistance in the eastern oyster. The objective of this research is to characterize disease tolerance in local, naturally selected (having experienced heavy disease pressure) oysters in relation to factors such as *P. marinus* infection, growth and genetics. Gene expression profiles have been characterized in oysters that have experienced heavy disease pressure and control oyster populations. Specifically, genes identified as putatively involved in oyster disease resistance were examined using quantitative real-time RT-PCR. One difference observed was with the gene expression patterns for *bcl-x*, a protein that suppresses the apoptosis pathway. In general, there appears to be an upregulation of apoptosis suppression (*bcl-x*) in oysters susceptible to Dermo. One interpretation is that oysters that have survived disease outbreaks have an ability to maintain hemocyte numbers at proper levels. Field trials with oysters that have experienced heavy disease pressure and control oysters are underway. Dermo presence has been detected in both groups, however, no significant differ-

ence in growth or mortality has been documented to date. This research is supported in part by the Cooperative State Research Education, and Extension Service, US Department of Agriculture, under Agreement No. 2003-38500-13505.

**METABOLIC CONVERSION OF PHYTOSTEROLS TO CHOLESTEROL IN ARTEMIA, A CRUSTACEAN MODEL ORGANISM.** Mark S. Dixon,<sup>1</sup> Hui Zhao,<sup>2</sup> Jose-L. Giner,<sup>2</sup> and Gary H. Wikfors.<sup>1</sup> <sup>1</sup>USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460; <sup>2</sup>State University of New York–ESF, Syracuse, NY 13210

Cholesterol is an essential component of all animal cell membranes. Vertebrate animals are able to synthesize cholesterol from simple precursors, but invertebrates lack this capability and must obtain cholesterol from the diet. Most phytoplankton cells have no cholesterol, but rather a diverse array of related compounds, referred to as phytosterols, as cell-membrane components. Thus, marine invertebrates that feed on phytoplankton are presumed to have abilities to modify some phytosterols to produce the cholesterol that becomes incorporated in their tissues as they grow, but identification of which phytosterols can be metabolized has not been accomplished previously. In this study, we used the brine shrimp, *Artemia*, as a model for crustaceans to explore metabolic capabilities for modifying microalgal phytosterols to cholesterol. Our approach was to enrich a cultured microalga with synthesized, purified phytosterols incorporating stable-isotope-labeled carbon at specific locations in the phytosterol structure, feed these enriched microalgae to brine shrimp, and determine the presence of labeled carbon in cholesterol extracted from the brine shrimp tissues.

The Cryptophyte RHODO (*Rhodomonas* sp.) has only one phytosterol, thus it served as our “blank template” for enrichment. Labeled phytosterols were dissolved in ethanol and incorporated into RHODO cells. The sterol-enriched RHODO was fed to *Artemia* in acrylic cubes filled with 4 liters of filtered seawater. *Artemia* were stocked at 5/ml and RHODO was initially added at 50,000 cells/ml. After 3 days the RHODO was increased to 100,000 cells/ml, and after 3 more days it was increased to 150,000 cells/ml. At the end of the 9-day trial, all *Artemia* were collected on a 100- $\mu$ m screen and frozen at  $-80^{\circ}\text{C}$ . *Artemia* tissues were extracted and tested for the presence of labeled cholesterol by Nuclear Magnetic Resonance Spectroscopy. Five of eight commonly-occurring phytosterols, including those with both 24-methyl and 24-ethyl substitutions, were converted metabolically to cholesterol by the brine shrimp. By contrast, 24-propylidenecholesterol was neither converted to cholesterol nor accumulated, and two phytosterols associated specifically with dinoflagellates were accumulated but not metabolized. These findings have relevance to the use of crustaceans as live feeds in

aquaculture food chains, as well as to natural trophic interactions between crustacean zooplankton and phytoplankton.

**BARNEGAT BAY SHELLFISH RESTORATION PROGRAM: YEAR 1, LEARNING FROM OTHERS.** Gef Flimlin and Cara Muscio, Rutgers Cooperative Research and Extension, 1623 Whitesville Rd., Toms River, NJ 08755

Following seven meetings of the International Conference on Shellfish Restoration, Rutgers Cooperative Research and Extension decided that all the proper pieces of the puzzle that are needed for an effective shellfish restoration project, including seriously diminished hard clams stocks, were in place in Ocean County, NJ and that the time was right to muster forces and begin the process. The program was quickly adopted by the Barnegat Bay National Estuary Program as an implementation project for the estuary revitalization process. Great strides were made in the first year following the leads of very successful programs in New York and Delaware with recruiting 35 volunteers, getting local funding of over \$50,000, having significant coverage from the press, documenting each phase of the process, and growing 600,000 hard clam seed in two upweller systems. Seed showed almost four-fold growth in 10 weeks. Mortality was much less than 1%. Seed was placed in ADPI bags in 4 locations for over-wintering and two small-scale research projects are ongoing. One relates to seed density in the over-wintering bags and the other is a study of glycogen content of the seed from the fall to the spring. The volunteer base has become well organized and is taking a leadership role to coordinate more facets of the overall process, including the formation of a 501 (c) (3) non-profit organization to support the program. This program, which is being conducted in conjunction with NJDEP Bureau of Shellfisheries, will continue with hard clams in the next three years as well as establishing 4 oyster reefs and populating them with oysters.

**A COOPERATIVE SHELLFISH HATCHERY MODEL: THE SOUND SCHOOL REGIONAL AQUACULTURE CENTER & THE GUILFORD SHELLFISH COMMISSION: SEVEN YEARS LATER.** Gabriel N. Geist, Bianca Farrell, Susan T. Murphy, and John J. Roy, Sound School Regional Aquaculture Center, 17 Sea St., New Haven, CT 06519

The Sound School Regional Vocational Aquaculture Center is a comprehensive high school that combines a scientific education with a hands-on training program centered on marine trades and industrial aquaculture. Students are taught a variety of industry-standard aquaculture techniques utilizing both finfish and shellfish in a variety of open and recirculating systems. In addition to classroom and laboratory experience, the Sound School offers both on- and off-site supervised occupational training in aquaculture techniques during summer vacations and after the typical school day

ends. Each student must complete 200 hours of this training each year.

The town of Guilford's Shellfish Commission (GSC) has a long history of wise resource management practice, support for utilization of current research and application of that information to meet the needs of the public. Local support for the GSC is strong and includes not only a well-informed public, but both fisherman and marina owners helping in their management efforts. A rise in license sales in the late 1990's combined with the decline of native oyster populations (due to disease) and hard clams (due to recreational fishing) has made the GSC receptive to alternative stocking efforts including aquaculture. A local support network of the public, businesses and education make the GSC a model program and a viable cooperative effort partner.

In the spring of 1999, the Sound School RVAC and the Guilford Shellfish Commission began a cooperative program to provide bivalve shellfish nursery technician training to high school students. Utilizing student constructed upwellers, the program has produced well over a million hard clams (*Mercenaria mercenaria* *x notata*) and close to a million eastern oysters (*Crassostrea virginica*) for stocking into recreational shellfish grounds in the town of Guilford. A unique blend of industry, education and government has provided shellfish technician training to more than 200 students while expanding the stocking effort and restoring populations of shellfish in Guilford. The program has experienced many 'growing pains' and has undergone several changes over the past few years in an effort to continue. The lessons learned from these experiences will help to provide guidance for the future of this program.

**FUNCTIONS OF GRANULAR AND AGRANULAR HEMOCYTES IN EASTERN OYSTERS FOLLOWING FLOW-CYTOMETRIC LIVE-SORTING.** Madeleine Gonçalves,<sup>1</sup> Gary H. Wikfors,<sup>2</sup> Jennifer H. Alix,<sup>2</sup> and Hélène Hégaret.<sup>3</sup> <sup>1</sup>Université de Bretagne Occidentale, Institut Universitaire Européen de la Mer, 29280 Plouzané, France; <sup>2</sup>USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460; <sup>3</sup>Department of Marine Sciences, UCONN, Groton, CT 06430

Hemolymph of the eastern oyster contains circulating cells, termed hemocytes, with between two and four distinct morphologies. Within the broad categories of "granular" and "agranular" cells, small and large sub-populations sometimes can be resolved. There are many questions about ontogeny and relationships between these cell types, and information on differences in function between different types of hemocytes is mainly provisional and incomplete. We are attempting to clarify functions in granular and agranular oyster hemocytes by physically sorting them with a BD Biosciences FACSVantage flow-cytometer and applying function assays (e.g., phagocytosis, respiratory burst) after sorting. Following initial, unsuccessful attempts to obtain sufficient numbers of

viable hemocytes after sorting, we modified test tubes used to collect sorted cells and changed the optical criteria upon which sorting was based. With these modifications, pure suspensions of viable, agranular hemocytes were obtained easily: these cells produced reactive oxygen species (ROS) when stimulated by bacterial extracellular products, but we were unable to detect phagocytosis of plastic microbeads by these cells with either flow-cytometry or fluorescence microscopy. Pure suspensions of sorted granular cells were more difficult to obtain; sort criteria based upon size and internal complexity yielded sorted cell populations comprised of both granular and agranular cells. To test the hypotheses that post-sort agranular cells were degranulated phagocytic cells, we "fed" a whole-hemolymph sample with plastic microbeads to distinguish phagocytic hemocytes, and sorted only the largest, most phagocytic hemocytes into a test tube. Again, a small population of agranular cells was found in the test tube, along with an appreciable number of free microbeads unassociated with hemocytes. Our interpretation is that some phagocytic, granular cells had discharged the microbeads and presumably the granules that distinguish them optically from true hyalinocytes during the sorting process. This preliminary work provides evidence that hyalinocytes in oysters are not phagocytic, but degranulated granulocytes may be indistinguishable optically by the flow cytometer from hyalinocytes.

**CONDITION INDEX STUDY OF QUAHOG POPULATIONS IN NARRAGANSETT BAY, RHODE ISLAND.** Jeffrey Grant and Michael A. Rice, Department of Fisheries, Animal & Veterinary Science, University of Rhode Island, Kingston, RI 02881

Greenwich Bay, on the western side of Narragansett Bay, Rhode Island is known for its active fishery for the northern quahog, *Mercenaria mercenaria*. Sustained catches of quahogs from Greenwich Bay and adjacent waters suggested that significant annual recruitment occurs, which raised the question as to the location of the parental stocks. Between March and October 20, 2005 quahogs were collected aboard the *F/V Thomi Boy* by bullrake on a monthly basis from 9 different sites around Narragansett Bay, including Greenwich Bay and adjacent coves, Providence River, Allen's Harbor, and conditional shellfishing areas A and B. The condition index of each quahog was determined to help characterize the overall health, and thus the overall potential fecundity of different stocks. The highest condition index values for most of the sites occurred in early June, however the cove areas reached a peak in late May. The mean condition index values ranged from a high of 116.2 in conditional area A in early June to a low of 54.6 in Warwick Cove in March. Conditional area A had the highest average condition index of 98.1, while Warwick Cove and Greenwich Cove had the lowest condition indices of 66.0 and 67.3 respectively. Based on the data collected, the dense assemblages of quahogs in the permanently closed areas may not be as reproduc-

tively healthy as other areas of Narragansett Bay. However, further research is needed to determine if a relationship exists between the condition index and overall fecundity of quahogs in coves and other areas of Narragansett Bay.

**EARLY STEAMER CLAM (*MYA ARENARIA*) RESTORATION EFFORTS IN TWO COASTAL PONDS IN OAK BLUFFS, MA (MARTHA'S VINEYARD ISLAND).** David W. Grunden and Danielle Ewart, Town of Oak Bluffs Shellfish Department, P. O. Box 1327, Oak Bluffs, MA 02557

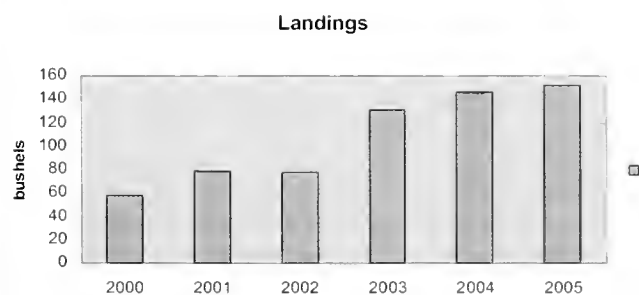
The investigation into this project began in 2001 when the Town of Oak Bluffs purchased an axial flow upweller and 200,000 seed (2–3 mm) steamer clams with financial assistance from the Massachusetts Department of Food and Agriculture.

Three trial restoration sites were chosen; two in Sengekontacket Pond and one in Lagoon Pond. One site in Sengekontacket Pond has remained open for harvest. The other two sites are alternated one opening every other year. All three sites are utilized primarily for recreational shellfishing, though occasionally a commercial shellfisherman does harvest from one of the sites in Sengekontacket Pond.

The first year the steamer seed was only grown in the upweller and broadcast seeded at 12–15 mm. The seed at this size dug into the sandy substrate within 15 minutes. The area was marked but ice removed the marker that first winter.

In subsequent years the Town of Oak Bluffs has been able to increase the amount of seed raised to 600,000 in 2005. The Town's Shellfish Department constructed sand-filled nursery rafts to allow for greater growout of the seed. The seed steamers are still removed from the upweller once they reach 12–15 mm, but are now transferred into the nursery rafts where they are held until late mid-September or early October. The seed measured 22.4–38.2 mm when they were planted out in the fall of 2005.

The fishing effort has been monitored by the Shellfish Department and we have seen an increase in the steamer clam landings from these areas since the project began. In 2003 an increase (67.9%) in the number of bushels landed was seen. The landing continued to rise in 2004 and 2005. When the 2002 season is compared to 2005 there has been a 94.87% increase in bushels landed.



**CORRELATION OF GONAD INDEX AND GONAD HISTOLOGY AS MEASURES OF SPAWNING ACTIVITY FOR THE BAY SCALLOP, *ARGOPECTEN IRRADIANS*, IN NANTUCKET HARBOR.** Valerie A. Hall, Robert S. Kennedy, Peter B. Boyce, W. Forrest Kennedy, Maria Mitchell Association, 4 Vestal Street, Nantucket, MA 02554; Erik Reinbergs and James Sjolund, Nantucket High School, 10 Surfside Road, Nantucket, MA 02554

As part of a larger study conducted by the Nantucket Maria Mitchell Association, the hermaphroditic gonads of 130 bay scallops collected on 18 dates from late July, 2004 through late September, 2005 were processed for histology. After sectioning and staining the paraffin-embedded specimens, microscopic examination was used to determine the stage of gametogenesis and to estimate the average oocyte diameter in the ovarian follicles. These data were correlated with the wet gonad index calculated from newly-dissected scallops collected in the field. A cohort of 25 adult scallops entering their third summer season (22–26 months after spawn) were chosen for special analysis because we were able to examine their gonads throughout their entire reproductive season, late February through late September, 2005. Preliminary comparisons had indicated that the average oocyte diameter exhibited a similar pattern as the gonad index in the wild population of bay scallops sampled in this study. Both parameters appeared to increase before and decrease while spawning occurred. However, further analysis indicated that the two values did not correlate well. Comparing oocyte diameter to gonad index in all 130 scallops, the correlation coefficient ( $r$ ) was 0.26. For the 25 adults studied through their entire reproductive season, the correlation coefficient ( $r$ ) was 0.31. Thus, the relationship between gonad index and mean oocyte diameter may not be a good indicator of spawning time in the Nantucket bay scallop. Nevertheless, the progression of the stages of gametogenesis could be traced through the histological studies, suggesting that the third-season scallops did spawn at the time indicated by the drop in gonad index. We need to develop alternate histological methods for determining a scallop's readiness to spawn, using a cohort of scallops from first season of reproduction to the end of their reproductive lifetime.

**NORTH CAPE SCALLOP RESTORATION PROJECT- LESSONS LEARNED FROM THE RESTORATION EFFORTS IN RHODE ISLAND'S SOUTH COUNTY SALT PONDS.** Boze Hancock,<sup>1</sup> James Turek,<sup>1</sup> Najib Lazar,<sup>2</sup> and John Catena,<sup>3</sup> <sup>1</sup>NOAA Restoration Center, 28 Tarzwell Dr., Narragansett, RI 02882; <sup>2</sup> Rhode Island Department of Environmental Management, Division of Fish and Wildlife, 3 Fort Wetherill Rd., Jamestown, RI 02835; <sup>3</sup>NOAA Restoration Center, One Blackburn Dr., Gloucester, MA 01930

In January 1996 the tug barge *North Cape* ran aground on the south coast of Rhode Island, releasing 828,000 gallons of No. 2 heating oil. The *North Cape* Restoration Program addresses mul-

multiple shellfish species including bay scallop (*Argopecten irradians*), along with a number of other natural resources injured by the oil spill. The Shellfish Restoration Program is a 5+ year, \$1.5 million effort to restore and enhance bivalve populations in Rhode Island's South County salt ponds and Narragansett Bay, involving federal and state agency staff and local community volunteers.

The initial phase of the scallop restoration project was to survey salt pond habitats, and release farm-raised seed broodstock in suitable habitat areas. Survival and distribution of the scallop releases were monitored during the following season using diver surveys, while the relative abundance, temporal and spatial distribution of settling spat were monitored using artificial spat collectors. Point Judith Pond, one of eight South County salt ponds was chosen as a pilot pond, and in October 2002, ~640,000 scallops (mean size ~30mm) were released. Monitoring during 2003 indicated very low survival and few spat were collected. Diver observations indicated that the high mortality was likely due to predation by crabs and starfish. As each of the semi-enclosed salt ponds presents a unique ecosystem, additional ponds were seeded in late 2003. A total of 2.1 million scallops were released to Potter, Green Hill, Ninigret and Quonochontaug Ponds. Diver surveys in 2004 indicated variable but low survival of these scallop releases. The highest survivorship was in Ninigret Pond where the surviving number was estimated at 9,300 (SE  $\pm$  5,500), or approximately 1.6% of the scallops released.

Low survival of released scallops prompted the establishment of a caged spawner sanctuary, as a second phase of restoration, in 2004. Approximately 10,000 scallop broodstock (1+ year class) were added to Ninigret Pond in spring of 2004. Broodstock were housed in 25, 2' x 2' cages of 1" plastic coated wire mesh, each containing four shelves supporting  $\frac{1}{2}$ " mesh pouches, with 100 scallops per pouch. A July 2004 spawning did not translate into a successful spat settlement, as determined using spat collectors. A modest, late season spatfall was recorded following a spawning in late September 2004, in the area of the broodstock. Scallop abundance surveys in 2005 estimated a substantial population increase, resulting from the 2004 spat-fall, of 132,000 ( $\pm$ SE 55,000). This population, along with an additional 10,000 caged broodstock, resulted in a spat-fall throughout Ninigret Pond during 2005 that was 4.5 times the 2004 level. The 2005 spat-fall suggests a similar increase in the abundance of scallops in 2006.

**TRANSPORT OF HARMFUL ALGAE VIA RELOCATION OF BIVALVE MOLLUSKS.** Hélène Hégaret<sup>1</sup>, Sandra E. Shumway,<sup>1</sup> Nicolas Salion,<sup>2</sup> and Gary H. Wikfors.<sup>3</sup> <sup>1</sup>Department of Marine Sciences, UCONN, Groton, CT 06430; <sup>2</sup>Produits Elabores Ploermeil, 56803 Ploermeil, France; <sup>3</sup>USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460

Aquaculture and restoration activities with bivalve molluscs, including oysters, clams, scallops and mussels, often include moving individuals from one body of water to another. Our study tests the hypothesis that harmful algae ingested by source populations of shellfish can be introduced into new environments by means of these shellfish relocations.

Several cultured strains of harmful algae, including *Prorocentrum minimum*, *Alexandrium fundyense* and *Heterosigma akashiwo*, were fed to various species of bivalve molluscs, *Crassostrea virginica* (eastern oyster), *Argopecten irradians* (bay scallop), *Mercenaria mercenaria* (northern quahog = hard clam), and *Mytilus edulis* (blue mussel) to assess the ability of the algal cells to pass intact through the digestive tract of the shellfish and subsequently grow. Shellfish were exposed to the algae at natural bloom concentrations, or to a control of a commonly-used microalgal food, *Rhodomonas* sp., for four hours. Clearance and feeding rates were measured; feces and pseudofeces were collected and observed under the microscope for presence or absence of intact, viable cells or temporary cysts of the algae. Ten bivalves of each species were also exposed for two days to a simulated harmful algal bloom at a natural bloom concentration. The algae were removed after two days of exposure, and bivalves were kept for two more days in ultrafiltered seawater. Biodeposits were collected and observed under the microscope after 24 and 48 additional hours to evaluate again the occurrence and condition of any algal cells. Subsamples of biodeposits were transferred into algal culture medium and filtered seawater and monitored microscopically for algal growth. Intact algal cells of *Prorocentrum minimum*, *Alexandrium fundyense*, and *Heterosigma akashiwo* were seen in biodeposits; generally these re-established growing populations in the first 24 hr after transfer, but less often after 48 hr. These data show evidence that transplanted bivalves may be vectors transporting harmful algae, but a "deuration" step may mitigate this risk.

**A PRELIMINARY, FEASIBILITY STUDY FOR COMMERCIAL AQUACULTURE OF *MERCENARIA MERCENARIA* UTILIZING LANTERN NETS VIA A LONG-LINE SYSTEM IN SOUTHWESTERN LONG ISLAND SOUND.** Jason Heilwell and Javier Torres, Bridgeport Regional Vocational Aquaculture School, 60 Saint Stephens Rd., Bridgeport, CT 06605

This project is designed to determine if Chinese-style lantern nets are a commercially feasible method for cultivating *Mercenaria mercenaria*. Dually, the data will also indicate if *Mercenaria mercenaria* will demonstrate a higher growth rate in the photic or aphotic zone. The lantern nets are suspended from a long-line to a depth of one meter below the water surface. The two hundred forty foot long-line is deployed approximately one-half mile off the coast of Jennings Beach, Fairfield, CT. The preliminary data analysis consisted of comparing weekly mean height measure-

ments of a five thousand, member test population. From September 13, 2005 until February 10, 2006 the mean height of the *Mercentaria mercenaria* increased from 17 mm. to 19.95 mm, demonstrating a mean height increase of 2.95 mm, indicating a daily growth rate of .0189 mm. The initial water temperature on September 13, 2005 was 16°C and as of February 10, 2006 was 3.2°C. In comparison, *Mercentaria mercenaria* cultivated via traditional clam beds demonstrated a daily growth rate of 0.09mm, at a temperature of no less than 15°C. Biofouling and weather related system damage are the initial operational drawbacks of the project. Future plans are to continue weekly sample measurements of the *Mercentaria mercenaria* and to continue observation of the system mechanics. Anticipated project revisions included the use of sea clams directly transferred from an upweller system to the lantern nets.

#### MONITORING THE RESTORATION OF THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA* IN RHODE ISLAND.

Jason Helyer,<sup>1</sup> Boze Hancock,<sup>2</sup> Jim Turek,<sup>2</sup> Najih Lazar<sup>3</sup> and John Catena,<sup>4</sup> <sup>1</sup>University of Rhode Island, N. Kingston, RI 02881; <sup>2</sup> NOAA Restoration Center, 28 Tarzwell Dr., Narragansett, RI 02882; <sup>3</sup> Rhode Department of Environmental Management, Division of Fish and Wildlife, 3 Wetherhill Rd., Jamestown, RI 02835; <sup>4</sup>NOAA Restoration Center, 1 Blackburn Dr., Gloucester, MA 01930

The North Cape Shellfish Restoration Program is rebuilding oyster populations in Rhode Island by seeding juvenile oysters. The program has seeded approximately 2.4 million juvenile oysters at six restoration sites since 2003. This study evaluated oyster restoration efforts by analyzing survival data, growth rates, and the presence of oyster disease in two cohorts of seeded oysters: 2003 and 2004.

Comparisons between survival, disease, and annual growth do not suggest any clear explanations for the decrease in oysters at two of the restoration sites, or the higher survival rates at the other four sites. Results suggest that disease is not affecting oyster survival as the two sites with the highest incidence of *Perkinsus marinus* also have the highest survival and growth rates. Future sampling done in both early summer and fall may provide better insight as to the temporal pattern of mortality as well as providing information about factors influencing oyster survival.

#### EVIDENCE OF LONG ISLAND SOUND OYSTER HEMOCYTE MICRONUCLEATION AS MEASURED BY FLOW CYTOMETRY. James B. Hughes. USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460

Eastern oysters (*Crassostrea virginica*) are found in coastal Connecticut marine habitats that are chronically affected by contaminants. The chromosomal damage in the form of breakage,

rearrangement and misdivision that results from this exposure, can be monitored indirectly using the micronucleus test. Micronuclei are small, intracytoplasmic pieces of chromatin that are either chromosome fragments that lack centromeres or complete chromosomes that lagged at anaphase due to spindle defects. Except for their small size, they resemble the major nucleus. The micronucleus test is an internationally recognized system for the detection of the clastogenic/aneugenic effects of compounds. Examination of micronuclear incidence has progressed from time-consuming microscopic analyses to a faster, more statistically thorough method through the use of flow cytometry.

Oysters were sampled during October–November, 2004 at 13 sites along the northern shore of the Long Island Sound from Darien, CT to Fishers Island, NY. Ten oysters were collected at each site and brought to Milford where they were held overnight prior to the collection of their hemolymph. Oyster hemolymph was drawn using 1.0 ml tuberculin syringes and placed in 1.5 ml microcentrifuge tubes on ice. A 100 µl aliquot of hemolymph from each oyster was transferred to 5.0 ml cytometer tubes along with 4.0 µl SybrGreen, 4.0 µl propidium iodide, and 100.0 µl of filtered sea water. Samples were analyzed using a BD Biosciences FACSCAN flow cytometer. Coefficient of variation of SYBRGreen fluorescence data were analyzed using Kruskal-Wallis one-way nonparametric analysis of variance test (STATISTIX 7.0).

Of the 13 sites, 8 were significantly different from each other, whereas 5 were statistically homogeneous. The Milford, CT site showed the lowest nuclear variance and was significantly less ( $p < 0.0002$ ) impacted than Scott's Cove in Darien, CT, which had the highest variance.

#### PRODUCTION METHODS FOR THE VENETIAN SOFT SHELL SHORE CRAB ("LE MOLECHE"). Richard C. Karney Martha's Vineyard Shellfish Group, Inc., Box 1552, Oak Bluffs, MA 02557; Robert D. Garrison, Wampanoag Aquinnah Shellfish Hatchery, 20 Black Brook Rd., Aquinnah, MA 02535

The production of "le moleche," the soft shell, green shore crab, *Carcinus aestuarii* is centered in the Adriatic seaport town of Chioggia, Italy at the south end of the Venice Lagoon. The fishery supports a local industry that employs over 220 fishermen and is valued at \$3.5 million. Fishermen receive about 35 euros per kilo for the molted crabs that are an important part of the local cuisine. Using methods that have changed little since the Middle Ages, crabs are caught in pound nets, segregated according to expected time to molting, and held to shed in submerged keepers. Key to successful production is the recognition of visual clues that signal the onset of ecdysis; knowledge traditionally passed from father to son. The application of the molting techniques used by the Italian industry to the production of soft shell green crabs using the species *Carcinus maenas* holds promise for development of a similar industry in the US.



**UPDATE ON NOAA'S NON-NATIVE OYSTER RESEARCH PROGRAM IN SUPPORT OF THE CHESAPEAKE BAY OYSTER EIS.** Jamie L. King, NOAA Chesapeake Bay Office, 410 Severn Ave., Suite 107, Annapolis, MD 21403

Federal and state agencies continue to prepare an Environmental Impact Statement (EIS) to evaluate the proposed introduction of a non-native oyster, *Crassostrea ariakensis*, in Maryland and Virginia waters. As a Cooperating Agency on the EIS, NOAA is conducting a 3-year, \$6M competitive grants program. This Non-native Oyster Research Program is currently in its second year, and is aimed at research priorities previously identified by the National Research Council and the Chesapeake Bay Scientific and Technical Advisory Committee. Although the research program's third and final year (FY06) will continue to fund projects through 2007, NOAA's objective is to facilitate the rapid transfer of new findings to the peer-reviewed literature to support EIS evaluations as the research progresses.

Toward this end, the NOAA Chesapeake Bay Office is sponsoring Quarterly Reviews to provide a forum for sharing and discussing the most current research findings and to ensure timely incorporation of research results into the EIS evaluations. The first review session was held in Spring 2005, and covered a variety of topics including *Crassostrea ariakensis* taxonomy and genetics; susceptibility to oyster diseases such as *Bonamia*, Dermo, and MSX; and basic life history and ecology. The Summer 2005 Quarterly Review focused on 'Aquaculture options: Biological and economic factors affecting aquaculture production of native and non-native oysters in the mid-Atlantic.' The Fall 2005 Quarterly Review addressed 'Potential for *C. ariakensis*–*C. virginica* interactions: Larval substrate selection, post-settlement competition, and fertilization interference.' Reports summarizing the preliminary findings presented at these review sessions are available at the NOAA Chesapeake Bay Office.

The NOAA Chesapeake Bay Office will also sponsor a special session on non-native oyster research at the 98th Annual Meeting of the National Shellfisheries Association. The session will culminate in publication of a special edition of the Journal of Shellfish Research. NOAA is funding the special session and journal volume to facilitate the rapid transfer of new findings to the peer-reviewed literature to support EIS evaluations as the research progresses.

**DIFFERENCES IN PROTEIN EXCRETION BY THE HARMFUL MARINE DINOFLAGELLATE, *PROROCENTRUM MINIMUM*, UNDER DIFFERENT CULTURE CONDITIONS, AS REVEALED BY SELDI PROTEOMIC TECHNOLOGY.** Yaqin Li,<sup>1</sup> Hélène Hégaret,<sup>2</sup> Shannon L. Meseck,<sup>1</sup> Gary H. Wikfors,<sup>1</sup> Mark S. Dixon,<sup>1</sup> and Jennifer H. Alix,<sup>1</sup>

<sup>1</sup>USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460;

<sup>2</sup>Department of Marine Sciences, UCONN, Groton, CT 06430

The dinoflagellate *Prorocentrum minimum* is listed among the "HAB" (Harmful Algal Bloom) phytoplankton because it sometimes is associated with harmful effects upon shellfish and other marine life in coastal waters. Studies with cultured isolates of this species have revealed a diverse array of bioactive effects, including inhibition of bacterial growth, allelopathic effects on other phytoplankton species; in feeding bivalve mollusks, altered feeding behavior, tissue pathologies, immunomodulation and mortality; and toxicity of cell extracts when injected into mice. Other studies have shown no bioactive effects of *P. minimum* cultures, leading to speculation that strains may differ in toxicity, or expression of toxicity is dependent upon physiological status of the population which is affected strongly by environmental and chemical conditions. Moreover, chemical identities of all toxic agents in *P. minimum* remain poorly known.

The present experiment was designed to investigate differential expression of proteins or peptides excreted into the media by *P. minimum* cells cultured under experimentally varied conditions: nutrient and gas replete, P-depleted, or air-restricted. Excreted proteins were analyzed using a relatively-new technology, SELDI (surface-enhanced laser desorption-ionization) time-of-flight mass spectrometry. SELDI utilizes protein chip array technology to separate proteins from complex mixtures with high resolution. Mass spectrometry is used to detect proteins that are selectively adsorbed to "chip" surfaces of different chromatographic surfaces while impurities are washed away. Of a total of over 20 different proteins and peptides detected in *P. minimum* culture media, the majority were present in all treatments, suggesting that these most-likely are normal, metabolic by-products. Gas restriction resulted in increased quantities of five proteins and decreased amount of six proteins compared to control. P-depleted cultures showed significant increases in four proteins and a decrease in one protein. A protein with M/Z 9235 was up-regulated in both gas-restricted and P-depleted treatments, relative to the control, suggesting the possibility that this is a general stress response. The changes in the protein profiles under different conditions may represent a number of physiological responses to protein level. For example, changes in proteins may involve cells switching from autotrophic to heterotrophic metabolic mode under air restricted condition. While under P-depleted situation, such changes in protein may involve the production of diketone, a breakdown product of carotenoid.

**PROGRESS IN ISOLATION AND EVALUATION OF NEW, PROBIOTIC BACTERIA FOR USE IN SHELLFISH HATCHERIES.** Hyun Jeong Lim,<sup>1</sup> Diane Kapareiko,<sup>2</sup> Steven Pitchford,<sup>2</sup> Jennifer H. Alix,<sup>2</sup> Shannon L. Meseck,<sup>2</sup> Mark S. Dixon and Gary H. Wikfors,<sup>2</sup> <sup>1</sup>Aquaculture Division, West Sea Fisheries Research Institute, Incheon 400-420, South Korea; <sup>2</sup>USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460



Hatchery production of shellfish seed is necessary to supplement natural recruitment which is constrained by various stresses including habitat loss, pollutant contamination, overfishing, and climate fluctuations. Bacterial diseases are considered to be a major cause of mortality in shellfish larviculture and hatcheries, however, over-use of antimicrobials can result in development of resistant strains of bacterial pathogens. The use of probiotics for disease prevention and improved nutrition in aquaculture is becoming increasingly popular as the demand for environmentally-friendly aquaculture grows. This study, therefore, has the objectives to isolate and evaluate efficacy of new probiotic bacteria and microalgae for use in shellfish hatcheries.

First, 26 probiotic-candidate bacteria were isolated from oysters, scallops, and a mass culture of green algae. Sixteen of these isolates inhibited known scallop-pathogen bacterial strains, B183 and B122, in disc-diffusion assays: 9 strains were from oysters, and 7 strains from scallops. Oyster larvae were exposed to these 16 probiotic candidates for 48 hours to determine possible positive effects on survival. Similar to the control (unchallenged oyster larvae), larval survival was >90% with all probiotic candidates. We then re-isolated the probiotic candidates from larval challenges and confirmed identities by gram stain, morphology, and Biolog, finding 7 distinct strains according to these criteria. Using a 12-well, microplate method, we performed further larval challenges to see if the probiotic candidates had a positive effect on larvae and suppression of mortality caused by the pathogen B183. Oyster larvae exposed to one probiotic candidate (Oy-15) had the highest survival rate, and further, addition of this probiotic candidate to oyster larvae challenged with the pathogen improved survival compared to the pathogen alone.

In addition to the bacterial strains, we also isolated 3 microalgae from Long Island Sound during oyster-spawning season using sorting flow-cytometry. We determined algal division rates to assess suitability for mass culture and eventual mixed culture with probiotic bacteria. Probiotic-bacteria candidates did not have negative effects upon the newly-isolated microalgae; however, *Isochrysis* sp., T-ISO, grew significantly slower when grown with the probiotic-candidate strain G1 than when grown bacteria-free.

In the future, we will confirm positive effects of our probiotic candidates during long-term rearing of oyster larvae and also determine nutritional value of mixed cultures of the new microalgal and probiotic bacterial strains. This study can be the basis for development of functional foods that incorporate probiotics in shellfish hatcheries.

**HYBRIDIZATION BETWEEN TWO DIFFERENT GENERA OF CLAMS, *SPISULA SOLIDISSIMA* AND *MULINIA LATERALIS*, AND EARLY GROWTH TRIALS.** Scott Lindell, Bethany Walton and Janice Simmons, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

The Atlantic surf clam (*Spisula solidissima*) and the coot clam (*Mulinia lateralis*) occupy different habitat niches along the eastern North American seaboard. The Atlantic surf clam supports a multi-million dollar fishery and has been the subject of pilot-scale commercial aquaculture. Among the interesting properties of the coot clam that have made it useful to biologists are its ease of culture, short generation time, and high rate of reproduction. Despite their apparent geographic and reproductive isolation we have cross-bred these two species in the lab. Larvae of coot clams and both hybrids (*Spisula* eggs x *Mulinia* sperm and *Mulinia* eggs by *Spisula* sperm) metamorphosed at 10 days at 20°C and were then reared on set screens and in sand substrate. Results of the first four months of growth of the hybrids and *Mulinia lateralis* have implications for reassessing the taxonomy of mactrid clam species, and may hold promise for a new aquaculture species.

**INVESTIGATION OF GENETIC VARIABILITY OF QPX, A PATHOGEN OF THE HARD CLAM, *MERCENARIA MERCENARIA*.** Qianqian Liu, Bassem Allam and Jackie Collier, Marine Sciences Research Center, Stony Brook University, Stony Brook, NY 11794

QPX (Quahog Parasite Unknown), a protistan parasite of the hard clam *Mercenaria mercenaria*, has caused significant mortality of hard clam stocks in many locations along the east coast of the United States and Maritime Provinces of Canada since the 1960's. In the summer of 2002, an outbreak of QPX disease occurred in New York, causing severe economical losses. In this study, we used different QPX isolates to evaluate the molecular genetic variability among QPX organisms obtained from different geographical locations. Investigated isolates included QPX obtained from New York clams in 2003 and 2004, the isolate ATCC 50749 initially obtained from Massachusetts clams in the late 1990's, and new isolates obtained from Massachusetts clams in summer 2005. Our data showed that in the 18S and 28S ribosomal genes, slight sequence variations exist in some isolates. At the same time, a complex pattern of sequence variations was found in the Internal Transcribed Spacer genes ITS1 and ITS2: the variability within a single QPX isolate is as great as that among the different isolates, suggesting that these multi-copy genes might not be a good choice to investigate QPX genetic variability on the strain level. Four mitochondrial protein-coding genes (NADH dehydrogenase subunit 1 and subunit 7; Cytochrome oxidase subunit I and Cytochrome b) showed no sequence variability among the four investigated isolates (two from NY 2003, 1 from MA 2005 and MA ATCC 50749). The significance of the variations observed in the 18S and 28S genes needs to be further studied.

**ASSESSMENT OF THE SETTLEMENT, GROWTH, AND SURVIVAL RATE OF THE EASTERN OYSTER (*CRASSOSTREA VIRGINICA*) ON STRINGS WITH SURF CLAM CULTCH AND STRINGS WITH SEA SCALLOP CULTCH.**

**Kady Marino, Karin A. Tammi, Christopher Davis, Johanna Fay, Timothy Scott and Dale Leavitt**, Center for Economic and Environmental Development, Roger Williams University, 1 Old Ferry Rd., Bristol, RI 02809

Traditionally, southern New England has been a major commercial source of oysters, specifically the eastern oyster (*Crassostrea virginica*). However, a series of events including hurricanes and disease has greatly harmed the industry in New England. The disease Dermo, caused by the protozoan *Perkinsus marinus*, is the most prevalent observed oyster disease in Rhode Island, and is largely responsible for low survival rates among oysters in Narragansett Bay. After a record low oyster yield in 1961, *C. virginica* began to make a comeback in Rhode Island waters in the early 1990s. A common method of oyster fisheries has been that of remote setting. The string method of setting has declined in popularity in recent years but has been the preferred larval setting mechanism of Luther H. Blount for decades. The aim of this research is to assess the benefits and feasibility of the string method by monitoring settlement, growth, and survival of eastern oyster spat when set on strings with surf clam and scallop cultch. The spat was monitored on Prudence Island through the summer and fall and was tested using RFTM and Ray's tissue assay for the protozoan *P. marinus*.

Our analysis of variance results indicated no difference in settlement for shell type (sea scallop or surf clam). However, larval settlement was significantly different depending upon horizontal and vertical placement of the strings within the tank, most likely due to light penetration and larval settlement behavior and air flow dynamics.

**CHANGES IN FEEDING AND EXCRETION RATES OF BAY SCALLOPS, *ARGOPECTEN IRRADIANS*, WHEN EXPOSED TO ELEVATED LEVELS OF AMMONIA IN A CLOSED SYSTEM.** Shannon L. Meseck, James C. Widman Jr., Lisa M. Milke, George Sennefelder, Mark S. Dixon, Diane Kapareiko and David J. Veilleux, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460

Good water quality is crucial to high survival and growth rates of bivalves held in closed or semi-closed rearing systems. As concentrations of nitrogenous waste increase in seawater systems, these compounds can become toxic. In a scallop nursery system, removal and addition of nitrogen can occur through numerous pathways. For example, removal of nitrogen can be attributed to phytoplankton uptake, while the main inputs of nitrogen are often through waste production from the scallops. The atmosphere can also act as an important source or sink of nitrogen depending on a

variety of factors, including concentration gradients and mixing. This study was designed to examine the sources and sinks of ammonia in a closed system used to grow bay scallops. The study further examined if high concentrations of ammonia would result in changes to those removal or input factors.

Bay scallops, ranging in size from 8 mm to 12 mm, were placed in buckets with 8 L of water for 24 hours, where they were fed every four hours, so that by the end of 24 hours they had been fed 2% of their live, whole-body weight. Every four hours, samples were taken to monitor the change in ammonia over time. The scallops were exposed to one of three levels of total ammonia: ambient seawater concentrations ( $<0.01 \text{ mg L}^{-1}$ ),  $1.5 \text{ mg L}^{-1}$ , and  $4.0 \text{ mg L}^{-1}$ .

For each of the buckets, removal rate by phytoplankton utilization, excretion from scallops, and other inputs/outputs were monitored. Results from the experiment suggest that the removal of ammonia via phytoplankton uptake did not change at higher ammonia concentrations. However, at the higher ammonia concentrations, there was a large loss attributable to atmospheric exchange, bacterial uptake, and/or adhesion of ammonia to surfaces of the containers. The input of ammonia via excretion from the scallops appears to remain similar at different ammonia levels. This short-term study suggests that the interaction of input and outputs of ammonia in a closed system is complex; however, scallop excretion and phytoplankton uptake of ammonia appear to be constant for a wide range of ammonia concentrations.

***TETRASELMIS CHUI* (PLY429) DIVISION RATE AND UPTAKE OF NITRATE AND PHOSPHATE AT EXPERIMENTALLY-VARIED PII.** Shannon L. Meseck, Jennifer H. Alix, Mark S. Dixon, Barry C. Smith and Gary H. Wikfors, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460

Mass cultures of phytoplankton are complex because growth is dependent upon a number of variables, including pH, light intensity, day length, nutrient availability, and temperature. In high-volume mass cultures of microalgae (e.g., 18,000-L tanks), at the NMFS Laboratory in Milford, a contaminating microalga can often be observed growing with *Tetraselmis chui* (PLY429—an algal strain used widely as an aquaculture feed) and can dominate the culture eventually. The succession of one algal species to another may be a consequence of differing light, nutrient, temperature, and pH requirements of the competing species. This study is the first part of an investigation on how pH can affect the succession of one algal species to another. The results presented focus on the effects that pH has on the growth of and uptake of nutrients by *Tetraselmis chui* (PLY429).

*Tetraselmis chui* cultures were grown aseptically in 2L carboys of F media at a temperature of  $19.5^{\circ}\text{C}$ , a light intensity of  $110 \mu\text{Einst. m}^{-2} \text{ s}^{-1}$ , with a light dark cycle of 16:8. The experiment was run at the following pH's: 6.23, 7.26, 8.07, 9.03, and 9.32 with

the initial pH's obtained by adding either hydrochloric acid or sodium hydroxide to the carboys. During the experiment, the pH was maintained at  $\pm 0.2$  pH units by the addition of carbon dioxide to the carboys. The pH did significantly affect the division rate ( $P < 0.01$ ) of PLY429. At the lowest pH, division rates were  $0.37 \text{ d}^{-1}$ ; whereas, division rate was  $0.88 \text{ d}^{-1}$  at a pH of 7.26. Fitting the data to a division rate curve revealed that the optimal growth of PLY429 occurs at a pH of approximately 7.8. Uptake of nitrate and phosphate per cell indicates that the pH had no significant effect on the utilization of nitrate ( $P = 0.71$ ); however, there was a significant effect of pH on the loss of dissolved phosphate in the cultures ( $P = 0.04$ ). Loss of dissolved phosphate from the media at pHs of 6.23, 9.03, and 9.32 were statistically indistinguishable and greater than at the other pH's. Unlike nitrate, phosphate can complex with other chemicals in the media (i.e., calcium, magnesium, sodium, and iron) and render it less bioavailable for phytoplankton uptake. Phosphate complex formation with these chemicals is dependent upon their relative concentrations and upon the pH of the media. Our data suggest that in the cultures in which pH was near 6 or above 9, phosphate may have reacted with other chemicals in the media to form complexes that were not available to the phytoplankton for growth. Thus, PLY429 may be at a competitive disadvantage if pH is outside of the optimal range, rendering it more susceptible to domination by microalgal contaminants.

**CHANGES IN ENZYMATIC ACTIVITY DURING EARLY DEVELOPMENT OF BAY AND SEA SCALLOPS, *ARGOPECTEN IRRADIANS* AND *PLACOPECTEN MAGELLANICUS*.** Lisa M. Milke, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460; V. Monica Bricelj and Neil W. Ross, National Research Council, Institute for Marine Biosciences, 1411 Oxford Street, Halifax, NS B3H 3Z1 Canada

Poor growth and survival of postlarval scallops is often observed in hatcheries during post-metamorphic development when they undergo marked morphological changes and may thus be particularly vulnerable to dietary deficiencies. Although stage- and species-specific differences in dietary requirements have been demonstrated, the mechanisms responsible for differential diet performance remain largely unexplained. While biochemical composition is central to assessing the nutritional value of a diet, an animal's digestive and assimilation capacity are equally important. Digestive capacity of sea and bay scallops may differ 1) over development in association with morphological changes, and 2) between species, as these two species are adapted to different environmental conditions and food supply.

To test these hypotheses, scallop postlarvae [initial shell height (SH)  $\sim 250 \mu\text{m}$ ] were reared on a mixed algal diet consisting of one diatom (*Chaetoceros muelleri*) and one flagellate (*Pavlova* sp. CCMP 459 or *Pavlova pinguis*) until 4–5 mm SH, and were sampled at intervals coinciding with major morphological changes.

A series of colorimetric assays were conducted to measure the specific activity of carbohydrases (laminarinase, cellulase and alpha-amylase), proteases (azocasein and cathepsin B) and lipase (esterase) in scallop tissues.

Preliminary findings do not indicate any trends in protease or lipase activity either over time or between species; however, clear patterns in carbohydrase activity were observed. Sea scallops exhibited higher laminarinase activity, but lower levels of alpha-amylase and cellulase activity than bay scallops, with the most pronounced changes in enzymatic activity occurring in both scallop species prior to attaining  $\sim 1.2$  mm SH. However, laminarinase activity was significantly higher than activities of either alpha-amylase or cellulase in both scallop species. This is consistent with previous reports of higher activities of laminarinase than alpha-amylase in adult sea scallop digestive glands. Interestingly, lower activities of laminarinase than either alpha-amylase or cellulase, were reported in digestive glands of adult bay scallops, further indicating that enzymatic activity may vary among scallop developmental stages and species. Our findings have implications for developing targeted diets that could optimize postlarval scallop growth and survival in commercial hatcheries and for understanding utilization of the natural food supply.

#### **DEVELOPMENT OF A SUBMERSIBLE MUSSEL RAFT.**

Dana Morse, Maine Sea Grant, Darling Marine Center, Walpole, ME 04573; Babe Stanley, Shaba Shellfish, 42 Old Mill Stream Road, Sullivan, ME 04664; and John Riley, University of Maine, Norman Smith Hall, Orono, ME 04469

In recent years, mussel producers in Maine have focused on floating rafts as the preferred method of suspension culture, augmenting the already substantial production of bottom-raised product. Raft culture produces a high quality mussel, and is relatively protected against predation from the large population eider ducks in the state. However, rafts have the twin drawbacks of potentially impacting navigation and scenery, and also of suffering damage from icy or otherwise extreme weather conditions.

In 2003, an idea came from industry to develop a submersible version of a mussel raft, and a group came together to support this idea, consisting of industry, engineering, conservation, homeowners and extension. The design incorporates a set of hollow pontoons with steel or aluminum cross pieces, and surface buoys. Air is pumped in or out of the pontoons, and the surface buoys regulate height below the surface, and absorb surface energy.

A scale model was tested in the Tow Tank at the University of Maine in 2004, and early results indicated that the concept had potential. Follow up to the model trials has included fundraising to build half- and full-scale versions of the rafts, and a full scale prototype is presently undergoing initial field trials. The advancement of this idea has implications for both the production methods used in the state, and its acceptance by potentially competing user groups.

# SEEKING THE MOST INTELLIGENT DESIGN: THE EARLY EVOLUTION OF OYSTER GARDENS IN MAINE.

**Dana Morse**, Maine Sea Grant, Darling Marine Center, Walpole, ME 04573; **Chris Davis**, Maine Aquaculture Innovation Center, University of Maine, Corbett Hall, Orono, ME 04469

A two-year oyster gardening program in Maine was initiated in 2004, and the inaugural class completed its work in September of 2005. Students received approximately 20 hours of classwork early in 2004, and began field activities in July of that year. Each student purchased equipment sufficient for floating culture of 1000 seed oysters, and was given assistance in the construction, deployment and maintenance of the floating bags and the oysters. Sites to accommodate these activities were obtained through the Limited Purpose Aquaculture License, as administered by the Maine Department of Marine Resources.

The program was successful in raising oysters, and the students have obtained an excellent education in relevant marine issues, as well as being enthusiastic consumers of their home-grown product. However, critical issues remain, if such an approach can be successful in the long-term in Maine. For example, how do we accommodate the continued interest in the program, without having an undue effect on space available to the commercial industry and to other users of the waterways? How can such programs be taught effectively, over large geographic distances? Discussions have taken place between the project partners, industry members and others, to ensure that the benefits of the oyster garden program in Maine are not outweighed by negative outcomes. One option, the development of community gardens, will be implemented for the next class, anticipated to begin in April of 2006.

# A HATCHERY EXPANDS TO HELP RESTORE BAY SCALLOP, *ARGOPECTEN IRRADIANS*, POPULATIONS AND FISHERIES IN NEW YORK.

**R. Michael Patricio**, Cornell Cooperative Extension of Suffolk County, 3690 Cedar Beach Road, Southold, NY 11971

The near total collapse of bay scallop populations and the historic fishery it supported, on Eastern Long Island, New York has prompted funding of a large scale restoration program. This four year effort will require raising production levels from 1 million to 10–20 million animals, annually. Although the hatchery has been in operation since 1991, this project will necessitate considerable expansion of all aspects of production. The hatchery has been using hatch algae culture for many years, however, to meet the demands of our restoration project, a continuous algal production system was put in place.

A Seasalter Continuous Algal Production Systems (SeaCAPS), system was installed to provide the volume of algae needed to increase the hatchery's production. This system was chosen because it could provide not only the volume needed for the project, but also a constant supply of higher quality algae compared to the batch culture system.

An expanded post-set growing facility was built to provide enough space for scallops that range from 250 microns to 2 mm. Juvenile scallops are placed on downweller sieves and receive coarsely filtered water which is free from macroplankton; a rotary drum filter was utilized to provide filtered (<100 $\mu$ ) seawater. Scallops (>2 mm) will be placed in spat bags for continued growth up to 10 mm. A portion of the 10+ mm scallops will be free-planted, while the rest will continue to grow in lantern nets to be used the following year as spawner sanctuary stock. It is expected that the expansion and improvement of our facility will meet the needs of the restoration program and help return the scallop fishery to Eastern Long Island.

# PROGRESS AT THE SOUND SCHOOL AQUACULTURE CENTER'S LOBSTER HATCHERY.

**Elyse R. Rabinoff, John J. Roy and Angel G. Santiago**, Sound School Regional Aquaculture Center, 17 Sea St., New Haven, CT 06519

Believed to be the first lobster hatchery operated by high school students in the nation, the Interdistrict Marine Educational Program's Instructional Lobster Hatchery (IMEP/ILH) is designed to provide high school students with an opportunity for the enhancement of knowledge in aquaculture and marine sciences. The development of "hands on" curricula assists students in applying lessons to "real world" situations. The program is funded by the Office of Urban and Priority School districts, the Connecticut State Department of Education as a Continuing Interdistrict Cooperative Grant and is administered by the Science Department at the Sound School.

The Lobster Hatchery Program was initiated in 2002 with technical advice coming from the University of Rhode Island. From the inception, the lobster hatchery program has been supported through a cooperative working relationship with the students and staff at Ella T. Grasso/Southeastern Regional Vocational Technical School in Groton. It was anticipated from the onset that the ILH would be a multiyear undertaking. Progress with propagation would be realized in incremental steps as more experience was gained with lobster husbandry.

During the past five years, the lobster hatchery has undergone several changes both in location and equipment design. The initial goals for the program have been met. Broodstock (*Homarus americanus*) have been successfully held, and viable young have been produced. Each year the students who are involved in the ILH make advances based on the work of the preceeding years' participants. Cannibalism is a major obstacle to successful lobster culture. Last year's primary objective was to have ten larvae survive past the fourth instar. During March and April of 2005, students collected, counted and sorted 3,852 viable larvae from the broodstock tanks. Young animals were fed enriched *Artemia* daily. The young were counted every other day and monitored for developmental advancement. Thirty-four animals had reached the fourth instar when a chiller malfunctioned killing all but two of the

juvenile lobsters. We were successful in keeping those animals alive until September of 2005.

This season we will incorporate what was learned last spring and implement some modifications in collection and culture techniques that should allow more individuals to reach the benthic stage. Our objective will be to have one hundred animals survive to reach the fourth instar. Within the next few years, it is expected that a portion of the juveniles produced will reach adulthood. As techniques continue to advance, the juveniles that are produced will be made available to both educational and aquacultural researchers. In the future, it is anticipated that the IMEP/ILII will become a reliable source of known-aged lobsters that can be used for research. We hope that these animals will help establish successful ventures in lobster culture that may one day serve to lessen the demands on the wild population of lobsters in Long Island Sound.

**THE ATTACHMENT STRENGTH OF *MYTILUS EDULIS* AND THE STRENGTH/LENGTH RELATIONSHIP.** James F. Reinhardt and Sean P. Grace, Southern Connecticut State University, 501 Crescent St., New Haven, CT 06515

A mussel's ability to remain attached to the substrate is contingent on its ability to attach at a greater strength than the maximum hydrodynamic force to which it is exposed. For mussels located in a bed, lift is often considered the hydrodynamic force most responsible for dislodgement. The force of lift is proportional to the surface area that the force acts upon, and can be approximated as the area of an ellipse. Lift is also proportional to the square of the velocity of water moving over the mussel. In order to accurately predict size based differences in the probability of dislodgement it is necessary to understand how attachment strength varies as size increases. This size-strength relationship must be put into the context of time, because both attachment strength and wave climate vary temporally. Climatologists have predicted an increase in hurricane activity, including an earlier start to the hurricane season. Given the predictions, it can be expected that greater wave energies may occur during the late summer months, when mussel attachment strengths are at their lowest.

To understand better how changes in wave climate may affect mussel dislodgment, attachment strengths of *Mytilus edulis* were measured at two different sites: 1) a protected site (Southport, CT) and 2) a wave exposed site (Jamestown, RI) monthly during spring tides, over the past year. There were significant differences in the attachment strength between sites and attachment strength covaried significantly with size. Information gained from this study is important in understanding how increased wave energies during late summer months may affect dislodgment rates of the blue mussel.

**EAST COAST SHELLFISH GROWERS ASSOCIATION—POLITICS, POLICIES AND PROGRAMS THAT AFFECT YOU!** Ed Rhodes, Executive Director, East Coast Shellfish Growers Association, Rutgers Cooperative Extension, 1623 Whitesville Rd., Toms River, NJ 08755

The East Coast Shellfish Growers Association (ECSGA) was born at a Milford Aquaculture Seminar four years ago. The ECSGA is now composed of about a hundred shellfish companies of all sizes from all of the Atlantic coastal states and includes a number of researchers and others with an interest in shellfish aquaculture. In 2005 the ECSGA was directly involved in a number of national issues that affect the shellfish industry and ECSGA members made four trips to D.C. to help educate Congress and Federal Agencies. In January during the annual all-coasts shellfish "Walk-on-the-Hill" 6 ECSGA members visited 17 senators and representatives over a 3-day period and discussed the need for a more coordinated approach by Federal agency interactions on shellfish issues, the erosion of aquaculture funding by both the National Oceanic and Atmospheric Administration (NOAA) and the Department of Agriculture, country of origin labeling definitions of "wild" and "farm-raised" shellfish, the EPA sewage blending proposal that would reduce standards for treatment plants, the need for good science to address the introduction of the non-native *Crassostrea ariakensis* species, and the need for Federal assistance in shellfish marketing and crop insurance.

Two months later we were back in D.C. following the acceptance by NOAA of the petition for an Endangered Species Act listing of the American oyster and helped arrange a House Resources Committee hearing on this issue where we offered testimony. In September we were in the capital supporting our Gulf coast brethren as they made their post-Katrina needs known to Congress and for the launch of the new Shellfish Caucus in the House of Representatives. We also visited key Congressional offices emphasizing repeal of the Byrd Amendment that has resulted in tariffs on American oysters exported to Canada. Finally, in December we attended a Pentagon briefing for the head of the Army Corps of Engineers to try and derail their march toward regulation of shellfish culture under provisions of the Clean Water Act. All of the issues above are still active and will be discussed in modest detail, along with some suggestions for actions that can be taken to keep the shellfish industry viable and growing.

**QUAHOG (*MERCENARIA MERCENARIA*) SPAWNER SANCTUARIES: DOES SIZE OR LOCATION MATTER?** Michael A. Rice, Department of Fisheries, Animal & Veterinary Science, University of Rhode Island, Kingston, RI 02879

Although the State of Rhode Island has undertaken projects since 1954 to relay quahogs (*Mercenaria mercenaria*) from pollution closure areas, many have argued that the numerous coves and inlets around Narragansett Bay that serve as a source of relay stock do in fact serve as *de facto* spawner sanctuaries. Adult qua-

hog densities in pollution closure areas have been reported to average as high as 190 individuals/m<sup>2</sup>, but population densities above 40 individuals/m<sup>2</sup> in the coves and other shellfishery closure areas are not unusual in Narragansett Bay. Several lines of evidence suggest that high density adult populations of quahogs in closure areas may be reproductively impaired and thus limiting their utility as spawner sanctuaries that replenish nearby shellfisheries. First, shell growth of quahogs in coves has been shown to be impaired, presumably due to crowding effects, and it is known that crowding affects soft tissue growth, including gonads, *before* shell growth depression in many bivalves. Second, data from Greenwich Bay show a gradient of quahog larval abundance higher in the open areas of the bay with adjacent coves nearly devoid of larvae throughout an entire spring-summer monitoring season. For example, in the middle of Greenwich Bay in 1993 peak larval abundance was in mid-June reaching 170 larvae/L, but in Apponaug Cove and Greenwich Cove that have high density adult assemblages >40 individuals/m<sup>2</sup>, a late July increase in larval numbers only reached a peak of 30 larvae/L. Subsequent data from the spring to fall of 1995 showed a down-Narragansett Bay gradient in mean seasonal quahog larval concentrations of 10.6, 16.0, 7.8, 5.4 and 3.6 larvae/L at Conimicut Point, Rocky Point, Warwick Point, Mount View/High Banks, and Wickford, respectively, with peak larval abundance on June 20 of 149.7 larvae/L and 197.4 larvae/L at Conimicut Point and Rocky Point, respectively. Collectively these data suggest that the quahog population in the large shellfishery pollution closure area in the Providence River and Upper Narragansett Bay may be the major source of larvae for much of Narragansett Bay, including Greenwich Bay. Third, recent 2005 data show that the condition index (CI) of quahogs from areas of high density, including Greenwich and Apponaug Coves, as well as the Providence River, to be substantially lower than in quahogs from areas that are actively fished, particularly the major conditional fishing areas in Upper Narragansett Bay. This growing body of evidence suggests that shellfishery pollution closure areas, especially in confined cove areas, may not necessarily serve well as *de facto* spawner sanctuaries. Partial harvest and relay of adult quahogs from the coves to managed sanctuary areas may be a good management alternative to increase overall spawning potential and subsequent quahog fishery recruitment.

**AN INVESTIGATION OF CILIATE XENOMAS IN *CRASSOSTREA VIRGINICA*.** Emily Scarpa and Susan Ford, Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Sciences Rutgers University, Port Norris, NJ 08349; Bruce Smith, New Hampshire Fish & Game, Durham, New Hampshire 03824; David Bushek, Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Sciences, Rutgers University, Port Norris, NJ 08349

Since the late 1990s, unusually high prevalences of xenomas have been noted during routine histological examination of oysters from Great Bay, New Hampshire. Xenomas are formed when in-

tracellular parasites accumulate within host cells, causing them to hypertrophy. Although in fish xenomas are commonly caused by microsporidians, in oysters xenomas are caused by ciliates, genus *Sphenophrya*, and are rare. Because they are macroscopically visible on gills, the marketability of infected oysters has been questioned.

In this study, samples were collected every fall from 1997 through 2005 and processed using normal histological procedures. In 2005, counts were also made of macroscopically visible xenomas. Prevalence varied according to site within Great Bay and also by year. In histological sections, it has increased notably since 1997, when only 1% of oysters were affected. In 2004, prevalence ranged from 33% to 82% between sites. Densities were mostly below 20 xenomas per histological section, but reached as high as 173. Macroscopically, samples from 2005 contained a mean 15.5 xenomas per oyster, ranging from 0 to more than 100.

The xenomas were located in gill water tubes and were often large enough to occupy the entire cross sectional area. They cause localized epithelial erosion and most likely impede water flow. Nevertheless, the histological appearance of the remaining tissues was not obviously affected and there was no clear correlation between oyster size and infection. There was an inverse relationship between the prevalence of *Haplosporidium nelsoni* (MSX) infections and that of xenomas, although the reason is presently unclear.

**STRATEGY FOR DETERMINING BIOCHEMICAL NUTRITION OF SHELLFISH BROODSTOCK CONDITIONING.** Ghazala Siddiqui,<sup>1</sup> Mark S. Dixon,<sup>2</sup> Joseph Choromanski,<sup>2</sup> David J. Veilleux,<sup>2</sup> and Gary H. Wikfors.<sup>2</sup> <sup>1</sup>Centre of Excellence in Marine Biology, University of Karachi, Karachi-75270, Pakistan; <sup>2</sup>USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460

The natural gametogenic cycle in oysters is closely associated with glycogen storage and subsequent synthesis of lipid during vitellogenesis. The lipid constituent of the egg is most affected by the quality of food provided to the broodstock and, thus, the deficiencies in essential fatty acids can alter fecundity and hatching rates and can trigger anomalies in the larvae. The importance of lipid and PUFA particularly docosahexonic acid (DHA; 22:6 *n*-3), eicosapentanoic acid (EPA; 22:5 *n*-3) and arachidonic acid (AA; 20:4 *n*-6) and sterols, 24-methylcholesterol or 24-methylenecholesterol, during egg and larval development have been recognized. Therefore, techniques have been employed to directly deliver or supplement the microalgal diets with these essential lipids used for broodstock conditioning.

The objective of this study is to evaluate the impact of fatty acid composition and sterol content of the algal diet on the fecundity, egg quality, hatching success and larval performance of *Crassostrea virginica* broodstock. For an initial experiment, the oysters

will be conditioned with unialgal diets of *Tetraselmis chui* and *Chaetoceros neogracile* and mixed diet of *T. chui* and *C. neogracile* (1:1, on dry weight basis). The fatty acid and sterol composition of these diets have previously been determined. Subsequently, an attempt will also be made to condition the oysters with diet supplemented with DHA, AA and sterols individually to confirm any specific requirements suggested by correlations found in the mixed-algal diet experiment.

#### **A PORTABLE, EXPERIMENTAL SHELLFISH-FEEDING SYSTEM: DESIGN AND VALIDATION OF OPERATION.**

**Barry C. Smith,<sup>1</sup> Hélène Hégaret,<sup>2</sup> Sebastian Saliou,<sup>3</sup> and Gary H. Wikfors.<sup>1</sup>** <sup>1</sup>USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460; <sup>2</sup>Department of Marine Sciences, University of Connecticut, Groton, CT 06340; <sup>3</sup>Pâtisseries Gourmandes SA, 22602 Loudeac Cedex, France

Environmental events, such as harmful algae blooms (HABs), typically do not occur at experimental research laboratories. Investigators are challenged to find ways to either bring the bloom or other environmental condition to the laboratory, or bring the experiments to the location of the given event. Compounding experimental location constraints, the duration of natural events can be very short. To address these realities, a water-exposure and phytoplankton-feeding system has been developed that is transportable to any location and can accommodate a variety of test organisms. Further, the system is readily adaptable to a variety of applications and experiment designs, from comparing responses of animals exposed to particulate and dissolved substances in seawater to conducting feeding studies with different phytoplankton suspensions.

A shipping tote forms the main structure of the exposure system. When set up for operation, twelve exposure trays reside inside the tote. Controlled water or culture flow is delivered to these trays through a bank of regulated flow meters. The original design supplied the flow meters with natural phytoplankton at ambient concentration, twice ambient concentration, and 0.2  $\mu\text{m}$ -filtered bay-water using a peristaltic pump and tangential-flow filter. By re-plumbing the four manifolds, other combinations of liquids can be metered to the exposure trays; an example was an experiment exposing shellfish to four different algal sources in four head jars delivered to the trays by gravity feed. The system has repeatedly performed reliably, within flow-rate tolerances, with a variety of plumbed arrangements, on two continents. Although validated with Eastern oysters (*Crassostrea virginica*), Suminoe oysters (*C. ariakensis*), and bay scallops (*Argopecten irradians*), any organism that can fit in the exposure trays could be used as a test subject. The controlled-flow regime also allows post-exposure treatment of water and organisms for quarantine. The system components pack into the shipping tote itself for secure shipment to any destination.

**A DEVICE FOR CONCENTRATING DINOFLAGELLATE CYSTS FROM SEDIMENT SAMPLES.** **Barry C. Smith<sup>1</sup>, Agneta Persson<sup>2</sup> and Gary H Wikfors.<sup>1</sup>** <sup>1</sup> USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460; <sup>2</sup>Smedjebacksvägen 13, SE-771 90 Ludvika, Sweden

Finding and enumerating protistan cysts from sediment in the natural environment is a tedious and time-consuming endeavor, generally involving sieving of large quantities of sediment and long hours of microscope searching. With increasing concern about harmful algal blooms (HABs), and the fate of the cysts produced at the later part of the bloom, development of improved methods to detect, enumerate, and determine viability of cysts is of increasing importance. Separating other sediment components would facilitate cyst identification and counting by concentrating them and removing obscuring debris. A system to mechanically concentrate cysts, undamaged, from the natural environment has been developed and tested. The system allows for quantifiable recovery of natural cysts for a variety of uses.

The "Particle Separator" is a rectangular trough with an inclined screen slanting up the long dimension. A sediment sample is suspended in water and delivered to the deep end of the trough by a peristaltic pump, and the suspension is circulated between a reservoir and the trough until all particles have settled. Each particle present has a unique combination of mass, volume, and surface coefficient of friction. Thus, similar particles will settle onto the screen at discrete locations along the length of the trough. Cysts are recovered from the section of the screen where they have accumulated. The system has been used to collect, identify, and quantify cysts from two locations. Further, a bivalve feeding study using natural dinoflagellate cysts has been conducted with cysts collected and concentrated from a sediment sample.

**EVALUATION OF SCALLOP GENETIC LINES FOR GROWTH, OVERWINTERING SURVIVAL AND PHENOTYPIC AND GENOTYPIC MARKERS.** **Sheila Stiles, Joseph Choromanski, Dorothy Jeffress, Steven Pitchford and Dionna Williams.** USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460

One of the major obstacles to bay scallop commercialization in the northeast US is overwintering survival. To address this issue, genetic lines of bay scallops, *Argopecten irradians*, are being selectively bred for improved growth and survival. One strategy is to increase growth rate to reach a marketable size prior to the onset of winter, thus bypassing the need to overwinter. Another approach is to select scallops for improved over-winter survival where temperatures often reach 0°C. A preliminary trial to assess overwintering survival from December to March of various bay scallop genetic lines of different sizes and ages in tanks with ambient flowing seawater resulted in survival ranging from 8.3%



to 45.5% in 2005. Lines were composed of  $F_1$  and  $F_2$  generation groups, selected primarily for growth, a self-fertilized inbred line, and lines with different phenotypic markers such as striped shells, which should reflect differences in genotypes. Mean sizes of scallops from all groups ranged from 15.68 mm to 48.84 mm. The highest percentage survival was in a low selected line with slower growth. This outcome suggests an inverse relationship between growth rate and survival. The next highest percent survival was in the inbred line, which could indicate that some inbred lines are better adapted than other groups for specific traits under certain conditions. More focused experiments are warranted to confirm these preliminary observations.

**OYSTER SET IN LONG ISLAND SOUND FOR CONNECTICUT (1997–2005).** Inke Sunila, State of Connecticut, Department of Agriculture, Bureau of Aquaculture, P.O. Box 97, Milford, CT 06460

Long Island Sound is a major growing area for eastern oysters, *Crassostrea virginica*. In Connecticut, oyster aquaculture is based on leased grounds, where oyster seed from natural seed beds is transplanted for grow-out. Only a small portion of oysters originate from hatchery-raised seed. Thus, a steady annual oyster set would result in a steady production of market oysters. However, commercial oyster set in CT occurs irregularly and often with intervals of several years, unlike in more southern estuaries. A major set in 1987 resulted in peak production during the following years and in 1995 Long Island Sound accounted for 94% of all cultured oysters in the eastern US.

Each fall the Bureau of Aquaculture collects oysters from the entire CT shoreline. One of the parameters measured is the set on oyster shells. During the fall survey the set from the previous summer is already visible to the naked eye. An average of 45 sites and 1370 shells are checked for the presence of set each year.

In 1997 and 1998 an epizootic of *Haplosporidium nelsoni* (MSX) affected CT's oyster stocks. MSX characteristically infects market size oysters first and seed in its second year. There was a heavy set in 1997, but the seed acquired MSX infection the next year. 1998 was also a year of relatively good oyster set, but the seed became infected as well. No significant oyster sets occurred during the following five years. Patchy sets were observed in some areas of no major commercial interest. Production decreased steadily because market size oysters decreased without further recruitment.

In 2004 a significant set occurred. It was a light set with on the average of only 15% of the shells surveyed set on. However, it landed in CT's major oyster growing areas south of Bridgeport, Norwalk and Westport. A good set also occurred in 2005, but mostly in the Milford-Branford area. No oyster set has been observed during the study years in the eastern end of the state between Clinton and Stonington. At the present time, oysters originating from the 2004 set have no significant disease prevalences.

Oysters originating from natural set need three to four years to reach market size. Without interference from a disease outbreak they are likely to form a new peak in CT's oyster production after a couple of years.

**BIOCHEMICAL COMPOSITION AND ADDUCTOR MUSCLE CELL SIZE OF TRIPLOID AND DIPLOID BAY SCALLOPS, *ARGOPECTEN IRRADIANS*.** Amandine Surier, Martha's Vineyard Shellfish Group, Oak Bluffs, MA 02557; Chester B. Zarnoch, Aquatic Research and Environmental Assessment Center, Brooklyn College, Brooklyn NY 11210

Triploidy which has been found to improve overall performance in many shellfish, is particularly interesting in scallops as the increased growth rate is associated with decreased gonad development and increased adductor muscle mass. Triploid's increase in somatic biomass is thought to be the result of energy reallocation from reproduction towards somatic growth or increased fitness due to higher heterozygosity. Another hypothesis is triploidy gigantism, which associates the increased size of triploid organisms with a larger cell size.

In July 2003, under funding from the Northeastern Regional Aquaculture Center, triploidy was induced in the bay scallop, *Argopecten irradians*. The experimental scallops, which tested 97% triploid in July 2004, were grown in bottom cages in Katama Bay, Massachusetts. Triploid wet adductor muscle indexes were 52% and 17% greater than diploids in July and October 2003 respectively.

In December 2003, triploid and diploid adductor muscle samples were sent to Brooklyn College for biochemical composition. Although the protein and carbohydrate contents were higher and lipid content was lower in triploid muscles the differences were not significant. In the spring 2004, survivors from the experiment were sampled for cell size analysis. A portion of the smooth adductor muscle from triploids and diploids was processed using standard histological techniques, photographed and analyzed for muscle cell size and number of cells/area using NIH ImageJ software. Adductor smooth muscle fibers of triploids were significantly wider than those of diploid controls. In contrast the number of muscle fibers per unit area was not significantly different between ploidy groups.

**ASSESSING IMPACTS OF SHELLFISH AQUACULTURE ON EELGRASS POPULATIONS IN EASTERN LONG ISLAND SOUND.** Jamie Vaudrey,<sup>1</sup> Tessa S. Getchis,<sup>2</sup> Robert Britton<sup>2</sup> and Jim Kremer.<sup>1</sup> <sup>1</sup>University of Connecticut, 1080 Shennecossett Road, Groton, CT 06340; <sup>2</sup>Sea Grant Extension Program, 1080 Shennecossett Road, Groton, CT 06340

Eelgrass (*Zostera marina*) is the dominant vascular plant of northern estuaries of the east and west coasts of the United States. Eelgrass beds provide critical ecological functions such as remov-



ing excess nitrates and phosphates and stabilizing fine sediments. Beds also provide critical habitat to a myriad of marine organisms including juvenile fish, shellfish, and crustaceans, among others. Eelgrass is considered an indicator of a “healthy” system and much attention has been focused on investigating potential causes of the loss of eelgrass and determining methods to minimize the decline in eelgrass abundance.

Bivalve aquaculture, specifically the utilization of submerged cultivation and depuration gear has been implicated as a potential source of negative impacts to eelgrass populations. However, shellfish aquaculture has also been shown to provide an equivalent or greater degree of ecosystem services as submerged aquatic vegetation.

This study was conducted to determine the type and degree of impacts and benefits that oyster depuration bottom cages have on eelgrass and surrounding water and sediment quality in order to provide producers and policy-makers with the scientific data needed to make informed decisions regarding the siting of aquaculture in Long Island Sound. Effects of the oyster cages were gauged by looking at reference sites (eelgrass areas without cages, bare areas with cages) and experimental plots (eelgrass areas with cages) in triplicate. Direct effects measured included: short shoot density, dry weight areal biomass, and growth rate. Indirect effects measured using water column and sediment samples included: benthic microalgae chlorophyll concentration, sediment percent organics, and water column chlorophyll concentrations. Water column profile measurements were recorded at all sites on the cage deployment and retrieval days (temperature, salinity, dissolved oxygen, turbidity, *in situ* chlorophyll). Video monitoring was utilized to document physical disturbance from the cultivation gear.

The water column at all three sites was vertically well mixed and no effect of the cages on water column characteristics was detectable. Sediment characteristics did not change as a result of the presence of the cages over the 2–3 week deployments. One deployment showed a statistically significant increase in sheath length at the eelgrass with cages site, indicating that the eelgrass was exhibiting either a stress response to reduced light availability or an increase in production due to higher nutrient availability in the pore waters of the sediment. Further deployments are needed to verify this response and determine the cause for the increase in sheath length. No other eelgrass indices showed a significant effect of the cages.

**COMMUNAL EFFECTS AMONG *ARGOPECTEN IRRADIANS*, *LITTORINA LITTOREA*, AND *ILYANASSA OBSOLETA*.** David J. Veilleux,<sup>1</sup> Sean Grace,<sup>2</sup> Ronald Goldberg,<sup>1</sup> and Dwight Smith.<sup>2</sup> <sup>1</sup>USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT, 06460; <sup>2</sup>Southern Connecticut State University, 501 Crescent St., New Haven, CT

In an attempt to investigate ways to control biofouling and sedimentation associated with aquaculture gear, two species of snail, *Littorina littorea* L. and *Ilyanassa obsoleta* Say, were added to bay scallops, *Argopecten irradians* Lamarck, being cultured in pearl nets (N = 15). The study site was in Milford Harbor, at the dock of the National Marine Fisheries Service, Milford Laboratory. The study was conducted over forty days, from June 29, 2004, to August 8, 2004. The total amount of fouling (as determined by weight), total growth of scallops and snails (as a measure of shell height and buoyant weight), sedimentation, mortality, and flow rate through the nets were analyzed. Results indicated that although there was no significant difference in amount of fouling, sedimentation, flow, and mortality associated with the snail treatments, there was a significant increase in the growth rate of scallops.

**SUPERIOR GROWTH OF TRIPLOID EASTERN OYSTER DEPENDS ON CULTURE ENVIRONMENT.** Yongping Wang,<sup>1</sup> Ximing Guo,<sup>1</sup> Gregory DeBrosse,<sup>1</sup> Rick Karney,<sup>2</sup> Paul Bagnall,<sup>3</sup> Jack Blake,<sup>4</sup> Susan Ford<sup>1</sup> and David Bushek.<sup>1</sup>

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The eastern oyster (*Crassostrea virginica*) faces three major diseases in the Northeastern region: MSX (caused by the parasite *Haplosporidium nelsoni*), Dermo (caused by the parasite *Perkinsus marinus*), and the juvenile oyster disease (JOD, caused by a bacterium). The development of a disease-resistant and fast growing stock would help the oyster culture industry. In a previous study, we have shown that hybrids between the Rutgers NEH strain (resistant to MSX and Dermo) and a commercial stock from the Flower's Oyster Company (FMF, known for its fast growth and JOD-resistance) outperformed either parental stock in yield under Dermo infections. To see if the hybrid advantage can be transferred to or amplified in triploids (3n) which grow faster than diploids (2n), we produced 3n hybrids between the two stocks and compared them to 2n hybrids, pure-line 2n and 3n controls. Four groups were produced in the summer of 2005: 1) 3nFN, 3n hybrids by crossing 2n FMF females and 4n NEH males; 2) 3nNN, 3n NEH by crossing 2n and 4n NEH; 3) 2nFN, 2n hybrids between 2n FMF females and 2n NEH males; and 4) 2nNN, 2n pure NEH control. The four groups were deployed at Cape Shore (CS) in New Jersey and two sites at Martha's Vineyard in Massachusetts: one in Katama Bay (KB) and the other in a hatchery at Edgartown Harbor (EH). Oyster spat were produced inside the hatchery using sterilized seawater and certified Dermo- and MSX-free before deployment at the two MA sites. At 3–4 months of age, 50–100 oysters were sampled from each group. Ploidy of each oyster was individually verified using flow cytometry and all three 3n groups

were 100% triploid. Triploids and diploids were compared in pairs between 3nNN and 2nNN, and between 3nFN and 2nFN. Growth at the KB site was significantly faster than the other two sites. Overall, the oysters were 24.7 mm in height at KB, 13.2 mm at EH and 12.6 mm at CS. Triploids were bigger than diploids at all sites, although the magnitude of difference varied considerably among sites. At the slow-growing sites (CS and EH), triploids were larger than diploids by 11–18% in shell height and 30–66% in whole body weight. At the fast-growing site (KB), triploids were strikingly larger than diploids—by 49–50% in shell height and 165–180% in whole body weight. This result demonstrates that superior growth in triploids depends on the culture environment. Triploids, when cultured in productive waters and systems, will bring considerable benefits to oyster farmers. The fact that triploids grow significantly faster than diploids at 4 months of age, long before sexual maturation, suggests that sterility is not an important factor contributing to the superior growth of triploids. The superior growth of triploids is likely caused by triploid gigantism, whose expression is dependent on culture environments. Triploids at KB also had fewer deaths from JOD than diploids.

**A COMPARISON OF BAY SCALLOP, *ARGOPECTEN IRRADIANS*, SURVIVAL AND GROWTH WHEN FED *TETRASELMIS CHUI* GROWN IN E OR F MEDIA.** James C. Widman Jr., Shannon L. Meseck, David J. Veilleux and Mark S. Dixon, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford CT 06460

Bay scallops, *Argopecten irradians*, were fed *Tetraselmis chui* grown in either E or f media. Phytoplankton concentrations were maintained at 8,000 cells/ml utilizing an automated computer control system. On a daily basis buckets were drained and filled with 15 L of 1  $\mu$  cartridge filtered seawater. Twice weekly, scallops were removed from the containers and placed in clean 15 L buckets. Shell height measurements were recorded weekly.

Scallop survival averaged 98% for both groups. There was no difference in survival of scallops fed *Tetraselmis chui* grown in either E or f media.

Urea concentrations after 24 hours were markedly different from one another. Urea ranged from 0.28–0.31  $\mu$ mol/L after 24 hours in scallops fed *Tetraselmis chui* grown in f media. Scallops fed *T. chui* grown in E media had urea concentrations ranging from 4.58–4.73  $\mu$ mol/L during the same time period.

Scallops fed *Tetraselmis chui* with an initial mean shell height of 7.9–8.0 mm grew to final mean shell heights of 12.3–13.5 mm 26 days later. Survival was high for scallops fed *Tetraselmis chui* grown with either type of media. After 26 days there were no significant differences ( $p > 0.05$ ) between scallops fed *T. chui* grown on either E or f media.

**DO VARYING *TETRASELMIS CHUI* CONCENTRATIONS AFFECT AMMONIA CONCENTRATIONS IN BAY SCALLOP, *ARGOPECTEN IRRADIANS*, CULTURES?** James C. Widman Jr., Shannon L. Meseck, David J. Veilleux and Mark S. Dixon, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford CT 06460

Bay scallops, *Argopecten irradians*, were grown in semi-static culture systems containing *Tetraselmis chui* at concentrations of 1,000, 3,500, 8,000 and 11,000 cells/ml. We wanted to determine whether phytoplankton concentrations would affect ammonia levels in the culture system. F media was used to grow the *Tetraselmis chui*. Microalgal cell concentrations were continuously monitored and maintained by an automated computer control system. Buckets were drained and filled with 15 L of 1  $\mu$  cartridge filtered seawater daily. Twice weekly scallops were removed from the containers and placed in clean 15 L buckets. Every week ammonia levels were measured over a 24 hour period.

Scallops grew from initial mean shell heights of 10.5–10.9 mm to final mean shell heights of 15.2–17.9 mm. Scallops grew at similar rates for the first 14 days. After 21 days, scallops grown at a cell concentration of 11,000 cells/ml were significantly smaller than those grown at 1,000 cells/ml. Throughout the experiment scallops grown at 1,000 and 3,500 cells/ml were not significantly different ( $p > 0.05$ ) from one another. Similarly, scallops grown at 3,500 and 8,000 cells/ml grew at similar rates ( $p > 0.05$ ). Scallops grown at the two highest concentrations, 8,000 and 11,000 cells/ml grew at similar rates ( $p > 0.05$ ). A previous study had shown no growth differences when scallops were fed varying concentrations of *T. chui* grown in E media.

Ammonia concentrations were not measured above 10  $\mu$ mol/L. There was no relationship between the ammonia concentrations observed and scallop growth rates.

In summary, ammonia levels were not affected by phytoplankton concentrations. Scallop growth was affected by phytoplankton concentration of *Tetraselmis chui* grown with f media.

**IMMUNE-STATUS ASSESSMENTS OF OYSTERS FROM THE 2004 ANNUAL STATE OF CONNECTICUT BUREAU OF AQUACULTURE SURVEY.** Gary H. Wikfors,<sup>1</sup> Inke Sunila,<sup>2</sup> Jennifer H. Alix,<sup>1</sup> April N. Croxton,<sup>1</sup> Hélène Hégaret,<sup>3</sup> and Shannon L. Meseck,<sup>1</sup> <sup>1</sup>USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460; <sup>2</sup>Bureau of Aquaculture, Department of Agriculture, State of Connecticut, Milford, CT 06460; <sup>3</sup>Department of Marine Sciences, UCONN, Groton, CT 06430

Every organism has a defense mechanism. When the capacity of the defense mechanism is exceeded, pathological changes follow. The State of Connecticut Bureau of Aquaculture has been conducting an annual autumn survey of oyster pathology for several years; the 2004 survey provided an opportunity to explore

possible relationships between pathology observed histologically and hemocyte immune-defense measurements made with flow-cytometry. Ten oysters from each of 14 sites selected from the survey were sampled for hemolymph and analyzed individually for descriptive hematology (numbers and percentages of different hemocyte types, percentage of dead hemocytes) and several immune functions (phagocytosis, respiratory burst, adhesion). Hemocyte parameters and histological findings were compared on an individual-oyster basis using multivariate statistics.

The strongest associations of hemocyte parameters were with intensity of parasitism by *Perkinsus marinus* (Dermo disease), as measured by the Mackin scale. Oysters with slight infestations (Mackin 0.5–1) had elevated granulocyte percentages and functions (phagocytosis, respiratory burst); whereas, circulating granulocytes and their functions were repressed with more-severe Dermo disease (Mackin 2 and above). These findings are consistent with the etiology of Dermo disease described previously: the initial stimulation of hemocyte functions represents induction of the defense mechanism to the parasite, and subsequent development of pathological changes accompanies exceedance of the defense mechanism. Other associations between pathology and hemocyte characteristics were less pronounced than those for Dermo because many oysters showed multiple pathologies, but several observations suggest possible future research topics, relative to immune status and pathology in oysters.

**LISTING A SPECIES AS THREATENED OR ENDANGERED—WHAT HAPPENED WITH THE EASTERN OYSTER PETITION?** Gary H. Wikfors, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460

A petition to consider the listing of the Eastern Oyster, *Crassostrea virginica*, as threatened or endangered pursuant to the Federal Endangered Species Act (ESA) was filed by Ecosystem Initiatives Advisory Services in January 2005. A positive initial finding (90 day finding), based upon evidence presented in the petition and existing information in possession of the NOAA ESA Program at the time of petition receipt, prompted creation of a Biological Review Team (BRT), consisting of representatives of NOAA and state resource agencies from throughout the US range of the species, to assess the status of the species. The process for determining whether a species should be listed is based upon the best available scientific and commercial information. The Status Review Report (SRR) is based upon an assessment of factors specified in section 4(a)(1) of the ESA that may be contributing to decline, including: 1) the present or threatened destruction, modification, or curtailment of its habitat or range; 2) overutilization for commercial, recreational, scientific, or educational purposes; 3) disease or predation; 4) inadequacy of existing regulatory mechanisms; or 5) other natural or manmade factors affecting the con-

tinued existence of the species. Finally, risk-of-extinction analysis must be completed by the BRT.

Sources of data that the BRT explored in the SRR included published scientific literature, presentations by invited experts, fishery and fishery-independent population assessments housed in various state and federal repositories, and a survey of state resource managers and academic experts from each coastal state within the species' range. The document will be peer reviewed, and as such, the SRR will represent the most comprehensive resource document for this species. The SRR will be completed, despite withdrawal of the petition prior to the decision date, thus, it is hoped that it will help inform coastal resource managers as they continue to manage and restore this species.

**INVESTIGATING THE OVER-WINTER MORTALITY OF HARD CLAM SEED, *MERCENARIA MERCENARIA*.** Chester B. Zarnoch and Martin P. Schreibman, Aquatic Research and Environmental Assessment Center (AREAC), Brooklyn College, The City University of New York, 2900 Bedford Ave., Brooklyn, NY 11210

Over-winter mortality of aquacultured hard clam seed is a significant problem for Mid-Atlantic and Northeast aquaculturists. Although protecting seed from predators improves survival, significant mortalities still frequently exceed 50%. This is often attributed to the severe temperatures of winter; however, this hypothesis has yet to be systematically investigated. We hypothesize that extended periods of low water temperature ( $<5^{\circ}\text{C}$ ) will result in reduced hard clam pumping, and thus an increase in the use of energy stores for metabolism. This would lead to an insult in physiological condition and cause mortality in the spring when water temperatures increase, food levels are low and metabolic demands are high.

In this study, seed (SL = 10 mm) were planted at two sites in Jamaica Bay, NY in the fall of each year between 2001–2004, to investigate the magnitude of over-winter mortality and to study physiological condition through the winter and spring. The data indicate that a mild winter results in negligible mortality. Similarly, a severe winter followed by a spring in which the rise in water temperature coincides with high food levels also results in low mortality. In contrast, significant mortality (up to 45% per sample) occurs in the spring following a severe winter, at a time when water temperatures are rising and food levels are low ( $<3\mu\text{g/L}$ ). During this period a rapid decline of carbohydrate content is observed, suggesting the use of energy reserves to maintain increased metabolic activity. Mortality occurs when carbohydrate levels fall below 10% of the tissue dry weight. Therefore, the timing of phytoplankton blooms and the increase in water temperature during the spring dictates the magnitude of over-winter mortality following a severe winter.

## ABSTRACTS OF TECHNICAL PAPERS OF THE JUVENILE OYSTER DISEASE SESSION

February 27–March 1, 2006

**A REVIEW OF BACTERIOLOGICAL STUDIES SUPPORTING *ROSEOVARIUS CRASSOSTREAE* AS THE ETIOLOGICAL AGENT OF JUVENILE OYSTER DISEASE IN *CRASSOSTREA VIRGINICA*.** Katherine J. Boettcher<sup>1,2</sup> and Aaron P. Maloy.<sup>1,3</sup> <sup>1</sup>Department of Biochemistry, Microbiology, and Molecular Biology, University of Maine, Orono, ME. 04469-5735. <sup>2</sup>Present address: 11208 Beechwood Pointe, Smithfield, VA 23430. <sup>3</sup>Present address: U.S. Geological Survey, Lake Ontario Biological Station, Oswego, NY 13126

By 1997, studies by other investigators had provided evidence for either a protistan or bacterial etiology for JOD. That year we conducted a study on Maine's Damariscotta River to distinguish between these two possibilities. Both a delay in JOD-onset and a significant reduction in cumulative mortalities were observed among oysters exposed to strictly antibacterial agents. In addition, there was a numerical dominance of a novel species of marine alpha-Proteobacteria in JOD-affected oysters. By the end of the recovery period, the levels of these bacteria in the survivors had dropped to <1% of total colonies. This species (named *Roseovarius crassostreae*) was subsequently found to be consistently and exclusively associated with JOD throughout the Northeast. Heavy colonization by *R. crassostreae* was also documented among a population of larger oysters with JOD-signs that were in otherwise good condition. Such observations argue against a role for *R. crassostreae* as a non-specific colonizer of terminally ill individuals. An attempt to reproduce JOD by exposure to *R. crassostreae* resulted in impaired feeding of challenged oysters, followed by mortality episodes during which the inoculating strain was reisolated from affected individuals. A later attempt to produce the entire suite of JOD-signs resulted in increased levels of conchiolin deposition among challenged animals (although without subsequent mortality). Finally, the presence of *R. crassostreae* was confirmed in a field population from Massachusetts one week prior to the appearance of gross signs and ensuing mortality. The cumulative evidence will be discussed within the current guidelines for proof of causation (e.g. Koch's postulates).

**A REVIEW OF MOLECULAR APPROACHES FOR EPIZOOTIOLOGICAL STUDIES OF JUVENILE OYSTER DISEASE AND DETECTION OF *ROSEOVARIUS CRASSOSTREAE* IN SITU.** Katherine J. Boettcher<sup>1,2</sup> and Aaron P. Maloy.<sup>1,3</sup> <sup>1</sup>Department of Biochemistry, Microbiology, and Molecular Biology, University of Maine, Orono, ME. 04469-5735. <sup>2</sup>Present address: 11208 Beechwood Pointe, Smithfield, VA

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We sought to develop genetic tools for the culture-independent detection of *Roseovarius crassostreae* and for understanding range expansion of JOD. Greater than 99.8% identity was found among the 16S rDNAs of isolates, but informative variation was detected within the 16S-23S rDNA internal transcribed spacer (ITS). Using restriction enzyme analysis and sequencing of the ITS region, a total of 11 genotypes were identified. In many instances, a single genotype was recovered from a given epizootic, but occasionally multiple genotypes were involved. Furthermore, phylogenetic analysis showed that diversification of the ITS did not map directly onto patterns of disease occurrence. Still, most genotypes were associated with specific years and/or locations. The data are consistent with the existence of indigenous *R. crassostreae* that become enriched at sites of oyster culture. A PCR assay was also developed for the direct detection of *R. crassostreae* from oysters. The assay was validated using closely related roseobacters, and no false positives were obtained from oyster samples. The assay has the potential to detect as few as 10 cells of *R. crassostreae* in oysters, and is equally or more effective than standard bacteriology in detecting the bacterium from field samples. Finally, in related studies we had shown bacterial microcolonies on the inner shell surfaces of oysters with JOD. To confirm that the bacteria were *R. crassostreae*, we used universal primers to produce a library of 16S rDNA clones from affected individuals. Sequence analyses confirmed the numerical dominance of *R. crassostreae* sequences and no other species was similarly abundant.

**JUVENILE OYSTER DISEASE: MICROFLORA FROM CONTROL AND INFECTED POPULATIONS OF AMERICAN OYSTERS (*CRASSOSTREA VIRGINICA*) ALONG THE ATLANTIC COAST.** Rocco C. Cipriano,<sup>1</sup> E. Jay Lewis<sup>2</sup> and Ana Baya.<sup>3</sup> <sup>1</sup>United States Geological Survey, National Fish Health Research Laboratory, 11649 Leetown Road, Kearneysville, WV 25430; <sup>2</sup>NOAA/NOS, Coastal Center for Environmental Health and Biomolecular Research, 904 South Morris Street, Oxford, Maryland 21654; <sup>3</sup>University of Maryland, College of Veterinary Medicine and Maryland Department of Agriculture, 8077 Greenmead Drive, College Park, MD 20742

The bacterial flora was evaluated from 11 populations of the American oysters (*Crassostrea virginica*) cultured in Virginia, New York, Massachusetts, Rhode Island, and Maine. Samples were obtained from both diseased and apparently unaffected oysters. The prevalence of juvenile oyster disease was based on mortality within a population and clinical signs of infection on individual oysters. Bacterial swabs were obtained from clinical internal surfaces with evident mantle recession, blackening, and conchiolin. Similar samples were obtained from the internal surfaces of shells from individuals that did not show clinical evidence of infection. Swabs were eluted (weight to volume) in sterile phos-

phate buffered saline and bacteria were cultured by dilution plate counts on medium containing 2.5‰ sea salts, 0.1‰  $\text{NH}_4\text{Cl}$ , 0.02‰ Na acetate, 0.03‰ peptone, 0.01‰ yeast extract, and 1.5‰ agar. When the results of the 11 sample sites were reviewed collectively, bacterial counts ranged between  $1.0 \times 10^6$  to  $4.1 \times 10^9$  colony forming units (cfu) per mL of sample. Motile aeromonads, pseudomonads, streptococci, *Comomonas terrigena*, *Acinetobacter* sp., and *Vibrio* spp. were commonly isolated. Because of their prevalence in both affected and non-affected oysters, none of the aforementioned organisms were considered putative etiologic agents. Even the vibrio isolates were obtained at nearly the same frequencies from infected and normal oysters. A total of 18 known vibrio species, as well as 26 unidentified species of vibrios, were obtained. Of these, *Vibrio alginolyticus* (45%), *V. splendidus* (42%), and *V. pelagius* (41%) were the most prevalent species. Unidentified vibrios were isolated so infrequently that they were not considered to cause disease.

**PATTERN, EFFECTS, AND REMEDIATION OF JUVENILE OYSTER DISEASE (JOD) IN THE DAMARISCOTTA RIVER ESTUARY, MAINE.** Richard Clime, Dodge Cove Marine Farm, Inc., P.O. Box 211, Newcastle, Maine 04553

Roughly eighty percent of Maine's cultivated American oyster production is located in the Damariscotta River. Common nursery techniques are deployment of electrically powered floating upwellers (FLUPSYS) for hatchery raised 2–2.5 mm seed (May–June), and graded transfer of 12–15 mm seed into floating bags (July–October) for end of season dispersal on shallow sub tidal bottom at 30–45 mm. Salinities in the areas of densest culture range seasonally from 23–31 ppt. Temperature maxima in August approach 24–27 °C, while winter minima, often under sea ice, can fall to –1.5 °C. Oysters will be metabolically active from April–November although most shell growth occurs from June through September.

JOD probably appeared in 1991 or 1992 although the effects were mild at first and hard to discern from other causes of mortality. By 1993 mortalities of nursery raised seed had surpassed 50% and in subsequent years would range from 50–80% of the seed crop. Symptoms appeared from late July to early August and were characterized by a cessation of growth at the 12–20 mm size, an extreme cupping of the lower valve, heavy interior shell concholin deposits, a general loss of healthy pigmentation, and eventual gaping before death. Distribution of diseased oysters within the floating bag nursery was non-random predominately affecting smaller, slower growing juveniles, but also showing a "locus centered" pattern within bag lines where some bags were fully involved while others were only lightly involved.

Attempts at remediation of the epizootic by our company began piece-meal with trial and error tests of the following strategies:

1) Split hatchery seed purchases between May and August deliveries.

2) Adopt use of FLUPSYS for small seed nursery culture (window screen tray and bag inserts previously used).

3) Geographically separate the FLUPSYS nursery from the floating bag nursery and where possible, locate disease free sites for FLUPSYS.

4) Undertake a formal selective breeding program choosing fast growing disease challenged oysters as broodstock.

5) Avoid overcrowding juveniles in the nursery while maximizing seawater circulation through the FLUPSYS and floating bags.

6) Expose hatchery juveniles (2–2.5 mm) and FLUPSYS juveniles (10–15 mm) to microbial probiotic colonization with lab cultures of *Stappia stellulata*, naturally occurring marine bacteria isolated from inside shell and mantle surfaces of healthy juvenile American oysters.

Other Maine companies may have tried a combination of different remedial strategies about which we are unaware.

By 2001, the course of the disease had lessened perhaps as a result of our remediation or perhaps it had simply run its course. Since then our nursery juvenile mortality from all causes has diminished to under 5% and overall yields of harvestable oysters have risen to 44% of purchased hatchery seed.

**A REVIEW OF JUVENILE OYSTER DISEASE (JOD) OUTBREAKS IN MAINE AND APPROACHES TAKEN TO REDUCE JOD INDUCED MORTALITIES.** Christopher V. Davis, Pemaquid Oyster Company, Inc., P.O. 302, Waldoboro, ME 04572 and the Maine Aquaculture Innovation Center, 5717 Corbett Hall, Orono, ME 04469, Paul D. Rawson, School of Marine Sciences, University of Maine, Orono, ME 04469, Bruce J. Barber, Eckerd College, 200 54th Avenue South, St. Petersburg, Florida 33711, Robert O. Hawes, Professor *emeritus*, University of Maine, Orono, ME 04669 and Scott C. Feindel, School of Marine Sciences, University of Maine, Orono, ME 04469

Outbreaks of Juvenile Oyster Disease (JOD) have been an ongoing concern for growers of Eastern oysters (*Crassostrea virginica*) in the northeastern United States since JOD's first documented occurrence in 1988. In Maine, JOD induced mortalities have resulted in juvenile crop losses exceeding in some cases 90%. A collaboration of industry growers and University of Maine researchers has led to the development of genetically selected broodstock and specific management strategies to help alleviate JOD impacts on Maine oyster farmers. Since 1985, the University of Maine's oyster selective breeding program has produced multiple lines of second and third generation broodstock that exhibit superior growth and JOD resistance compared to unselected controls. Concurrently, studies relating size-dependency to rates of JOD induced mortality have led oyster farmers to develop management strategies that maximize growth prior to and following midsummer epizootic periods when high JOD induced mortalities tend to occur.

**JOD INVESTIGATIONS BY RUTGERS AND SUNY, STONY BROOK, 1991-1994: EPIZOOTIOLOGY, HISTOPATHOLOGY AND BACTERIOLOGY.** Susan Ford, Haskin Shellfish Research Laboratory, Rutgers University, Port Norris, NJ 08349

In 1988, a producer in Maine sent a batch of juvenile oysters to the Haskin Shellfish Research Laboratory (HSRL) for pathological examination. The oysters had the classical symptoms of what would become known as Juvenile Oyster Disease (JOD)—the organic deposit on the inner shell—but no histologically detectable pathogen. Although this may have been the first recorded outbreak of JOD, concerted investigations of the disease did not begin until 1991, after extensive juvenile oyster mortalities occurred on Long Island, New York. That year, studies were conducted by HSRL and SUNY Stony Brook and by Battelle Ocean Sciences, at the Frank M. Flower Co. at Oyster Bay, NY, on the north shore of Long Island. They documented 1) the timing and extent of mortalities; 2) that they occurred at temperatures  $\geq 21-22^{\circ}\text{C}$ ; 3) that reducing densities also reduced mortalities; and 4) that lesions, but no obvious pathogen, were detectable in the gills and mantle of affected oysters by either light or electron microscopy, although bacteria were observed on and within the deposit. This study also recorded a link between phytoplankton blooms and the occurrence of lesions. In 1992, a larger study was undertaken that involved 8 sequentially spawned cohorts deployed over a several-month period at sites in and offshore of Oyster Bay, and a separate series deployed at the Blue Points Hatchery on the south shore of Long Island. Wild set also developed JOD symptoms when exposed in Oyster Bay. Regardless of age or size, JOD symptoms and subsequent mortality began about the same time in all groups at Oyster Bay, but never appeared at Blue Points. Oysters  $>25$  mm developed shell-deposit symptoms, but experienced low mortalities ( $<30\%$ ) compared to smaller oysters in which losses were 60 to 90%. Histologically detected lesions on the mantle were highly correlated with shell deposits and mortality, and were present about 2 weeks before mortality began. Results offered the FMF Co. a means to control the disease: earlier hatchery production so that the oysters would be  $\geq 25$  mm when the “window” of exposure to the JOD agent(s) was present. At about this time, we became aware of research in France that implicated a marine bacterium in the genus *Vibrio* in Brown Ring Disease (BRD) of Manila clams. The BRD symptom, like that of JOD, is an organic deposit on the inner shell. Consequently, HSRL and SUNY Stony Brook embarked upon a third project in collaboration with the FMF Co. It was conducted in 1993–94 and focused on determining the role, if any, of *Vibrio* bacteria and of plankton blooms in JOD. Field sampling of three cohorts showed an exponential increase in total *Vibrio* counts just before the onset of JOD symptoms, and that the *Vibrio* increases were preceded by plankton blooms. Several *Vibrio* isolates were used in challenge experiments, and al-

though several caused mortality, none consistently reproduced the JOD shell deposit symptom. A final study was conducted in 1994 when Dr. Christine Paillard, the French investigator who discovered the cause of BRD in Manila clams spent several months at HSRL. She demonstrated that mantle fluid of affected oysters caused JOD symptoms when injected into the shell cavities of experimental oysters. She also isolated a number of bacterial species (genera *Vibrio*, *Aeromonas*, and *Pseudomonas*) that were systematically and predominantly present only in individuals with JOD symptoms. None, however, produced JOD symptoms in challenged oysters. The conclusion of these studies was that JOD was most likely caused by one or more bacterial species, perhaps associated with plankton blooms, which affected principally oysters in crowded culture situations and that outbreaks were probably triggered by temperature.

The Northeastern Regional Aquaculture Center funded most of this work.

**TESTING DISEASE RESISTANCE IN OYSTERS: EXPERIMENTAL INFECTIONS WITH BACTERIAL PATHOGENS IN *CRASSOSTREA VIRGINICA* LARVAE AND SPAT.** Javier Gómez-León, Luisa Villamil, Rachel Hadley and Marta Gómez-Chiarri, Department of Fisheries, Animal, and Veterinary Science, University of Rhode Island, 20A Woodward Hall, Kingston, RI 02881

Culture of the American oyster (*Crassostrea virginica*) is a traditional activity that has great economical importance in the East Coast of USA and the Gulf of Mexico. Globally, shellfish production is often affected by bacterial pathogens, mainly *Vibrios*, which lead to high mortality rates in shellfish hatcheries. Another bacterial disease that has heavily impacted oyster culture in the Northeast US is Juvenile Oyster Disease (JOD), thought to be caused by *Roseovarius crassostreae*. In the present study, bacterial isolates that caused important mortalities in Pacific oyster larvae, RE22 and RE101, as well as an isolate from JOD-affected oysters (CV919-312) were used to perform experimental infections of oyster larvae and spat in order to determine differences in the susceptibility to bacterial infection of three oyster lines: a local Rhode Island line, a line resistant to Dermo and MSX (NEH), and a line resistant to JOD (FMF). All bacterial isolates tested were able to induce significant mortality in larvae and spat of *C. virginica*, reaching mortalities ranging from 50–100%. Differences in susceptibility between the lines were observed, with the NEH line showing the highest survival. Infected larvae exhibited abnormal circular swimming movements on their sides and deformed velum with cilia clumping. Factors affecting survival included temperature and size of the oysters. This research may provide a useful tool to test for disease resistance mechanisms in oysters.

**INVESTIGATING THE ETIOLOGY OF JUVENILE OYSTER DISEASE.** Geoffrey C. Horwitz<sup>1</sup>, Shawn Polson<sup>2,4</sup>, Mats Lundqvist,<sup>2,4</sup> Sara Polson,<sup>2</sup> Julie Higgins,<sup>2</sup> Earl J. Lewis,<sup>3</sup> Dorothy Howard<sup>3</sup> and Cheryl M. Woodley.<sup>2,4</sup> <sup>1</sup>Trinity University, One Trinity Place, San Antonio, TX 78212; <sup>2</sup>NOAA, National Ocean Service, CCEHBR, Hollings Marine Laboratory, 331 Fort Johnson Rd, Charleston, SC 29412; <sup>3</sup>NOAA, National Ocean Service, Cooperative Oxford Laboratory, 904 S. Morris St., Oxford, MD 21654; <sup>4</sup>Medical University of South Carolina, Marine Biomedical and Environmental Sciences, Charleston, SC 29412

Juvenile Oyster Disease (JOD) was first observed in 1984 and was responsible for the loss of over 95% of the seed oysters deployed for grow-out in the late 1980's and early 1990's. Hatchery owners have circumvented the disease primarily through improvements in management practices and the development of brood stock less susceptible to JOD. However, because no definitive causative agent has been identified and no simple diagnostic or prevention techniques currently exist, the disease remains responsible for a significant number of oyster deaths and financial losses each year. In this study, mantle tissue from both healthy and diseased (as assessed by the presence of conchiolin) oysters was screened for the presence of the suspected bacterial pathogen, *Roseovarius crassostreae*, and new candidate pathogen-associated protists by examining 16S and 18S ribosomal sequences, respectively. Similar methodologies were also used to examine diagnostic inclusion bodies which had been isolated from histological samples of mantle tissue using laser capture micro dissection. A strong relationship was found between both the presence of *R. crassostreae* and the candidate protist *Paranophrys magna*, but not in the second protist examined, the suctorian *Prodiscophrya sp.* in oysters with diagnostic signs of Juvenile Oyster Disease. While recent literature has ignored the possibility of a protozoan etiology, these results indicate that a protist component of the disease is not only possible, but very likely.

**OBSERVATIONS ON AN EPIZOOTIC OF JUVENILE OYSTER DISEASE (JOD) IN KATAMA BAY IN EDGARTOWN, MASSACHUSETTS.** Richard C. Karney, Martha's Vineyard Shellfish Group, Inc., Box 1552, Oak Bluffs, MA 02557; John C. Blake, Sweet Neck Farm, Box 1468, Edgartown, MA 02539

In 2003, oyster farmers in Katama Bay on Martha's Vineyard suffered losses of up to 99% of their seed oysters due to an epizootic of Juvenile Oyster Disease (JOD). This was the first confirmed JOD mortality on the farms that have operated in the bay since 1996. The first manifestation of the disease was observed in early August 2003 as a slowed growth rate in 12 mm (average) seed in tidal upweller nurseries. Mortalities and shell deformities were evident by mid September. Juvenile oysters from 2002 cultured nearby in bags on racks also showed slow growth and mortalities in 2003. These oysters were about 50–60 mm when im-

pacted and had mortalities as high as 50%. Some of these 2002 oysters survived the disease but continued to grow slowly to market size and showed a distinctive raised, brown ring on the interior shell surface. JOD related mortalities continued to be observed in oyster seed cultured in the bay in 2004 and 2005 but with reduced intensity. There is evidence for differential survival tied to lineage, and the use of resistant seed stocks is believed to have been key to the improved production in 2004 and 2005.

**A SUSPECTED CASE OF JUVENILE OYSTER DISEASE AT GREAT BAY, NEW HAMPSHIRE IN 1984.** Richard Langan, Cooperative Institute for New England Mariculture and Fisheries, University of New Hampshire, Durham, NH 03824

A commercial oyster farm in the Great Bay Estuary, New Hampshire experienced heavy mortalities in a cohort of year one Eastern oysters (*C. virginica*) in 1984. One hundred thousand cultchless oyster seed, purchased from a hatchery in Maine, were deployed in stacked trays in May 1984. The trays were suspended from a raft in near-surface waters and maintained at densities equivalent to 80%–100% of the surface area of the trays. The oysters grew from their initial size of 5 mm to approximately 25 mm by September with virtually no mortality. In late September, growers began to notice some gaping oysters with some chalky deposits on the shells. In October, mortalities began to appear and total mortality by the end of November was approximately 30%. Specimens of affected oysters were sent to a shellfish pathologist at the University of Rhode Island. Diagnosis revealed inflammation and degeneration of mantle tissue and conchilin deposits on the shells, however, no pathogenic organisms were isolated. The surviving oysters were transferred from trays to wire mesh cages and placed on bottom over the winter. When retrieved in the spring of 2005, approximately 50% of the oysters were dead. Conchilin deposits were found on the shells of the dead oyster as well as some that had survived. After removing the shells of dead oysters, the survivors were redeployed in bottom cages and grown out to market size over the next two years without any significant mortality. Subsequent year classes of seed that were purchased from the same hatchery were grown in the same location using similar methods without incident.

**JUVENILE OYSTER DISEASE (JOD) STUDIES 1991-2005.** Earl J. Lewis,<sup>1</sup> C. Austin Farley,<sup>2</sup> Dorothy Howard,<sup>1</sup> and Tim K. Mangel.<sup>3</sup> <sup>1</sup>NOAA, National Ocean Service, Cooperative Oxford Laboratory, 904 S. Morris St., Oxford, Maryland 21654; <sup>2</sup>NOAA, National Ocean Service, Cooperative Oxford Laboratory, 904 S. Morris St., Oxford, Maryland 21654—Retired; <sup>3</sup>University of Maryland, Department of Biology, College Park, Maryland 20742

Cultured oysters, *Crassostrea virginica*, moved from Maine to Great Bay, New Hampshire experienced mortalities in 1984.



Shells of dead oysters were unique in the high prevalence of conchiolinous deposits on the interior of the shell. By 1990, cultured oysters throughout the northeast region experienced mortalities in first year oysters 10–30 mm in shell length. The disease, juvenile oyster disease (JOD), only caused mortalities in cultured *C. virginica* juveniles although juvenile hard clams, *Mercenaria mercenaria*, oysters, *Ostrea edulis*, and other invertebrates were cultured in or inhabited nearby waters. All genetic strains of *C. virginica* evaluated were susceptible to JOD and the disease was found to be the same in oysters from Maine South to New York. Multi-year studies showed oysters experience fast growth and feed well until an abrupt cessation of growth and the start of mass mortalities. Affected oysters show no new shell growth and produce conchiolinous deposits on the interior shell that partially or completely encircle the oyster. As the disease progresses, oysters develop mantle recession and the adductor muscle of some oysters migrate over areas of conchiolin and loses its ability to tightly close the shell. In some cases oyster meats detach and float free of the shells. Mortalities of 60–80% are not uncommon and may approach 100% in some groups. Survivors of JOD often have shell checks, thickened ridges or growth rings that correlate with the size of the oyster at disease onset and perhaps recurrence of the disease. Bacteriological studies of mantle tissue, shell lesions, and shell fluid showed comparable bacterial flora and counts in infected and uninfected oysters from numerous locations. Protistan cultures commonly yielded parasitic ciliates, such as *Mesanophrys* sp., *Metanophrys* sp., *Paranophrys* sp., and a suctorian *Endosphaera* sp., that were isolated only from JOD infected oysters and water from JOD affected sites. When examined by histology, affected juvenile oysters have focal mantle lesions containing intracellular inclusion bodies that possess a single nucleus with condensed Feulgen-positive material or one large and a smaller secondary Feulgen-positive body like the macro and micro nucleus of a ciliate. Mantle lesions progress in size and number and ultimately form ulcerations. Mortalities were shown to peak one week after mantle lesions peak and begin to drop one week after lesions begin to resolve. A battery of special stains revealed no further information on identification of the small round bodies or cause of the disease. Electron microscopy showed the intracellular bodies to contain what appear to be nuclei with condensed chromatin, but usually lacking a nuclear membrane.

**JUVENILE OYSTER DISEASE (JOD)-TRANSMISSION, THERAPEUTIC TREATMENT, AND DISEASE RESISTANCE STUDIES.** Earl J. Lewis,<sup>1</sup> C. Austin Farley,<sup>2</sup> Dorothy Howard,<sup>1</sup> David Relyea,<sup>3</sup> Joseph Zahltla,<sup>3</sup> and Gregg Rivara.<sup>4</sup>

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Early studies suggested juvenile oyster disease (JOD) was caused by an infectious agent rather than a genetic, nutritional, toxin, or environmental condition. In addition, the movement of oysters from JOD-infected locations often appeared to cause the outbreak and continuance of disease at previously unaffected culture facilities. Field studies at Long Island New York sites routinely showed disease onset was three to four weeks after water temperatures exceeded and remained  $\geq 20^{\circ}\text{C}$ ; mortalities then continued throughout the growing season. Multiple laboratory experiments conducted over several years showed the disease was transmissible to different genetic stocks of oysters, *Crassostrea virginica*, using infected oysters and size specific fractions of material filtered from growing water at infected culture facilities by standard bag filters (5–100  $\mu\text{m}$ ). The disease was transmitted at salinities above 18 ppt, the onset of disease was three to seven weeks depending on water temperature, and the 10–25  $\mu\text{m}$  size fraction of filtered material produced the heaviest mortalities and highest prevalence of conchiolin. Transmission rarely occurred from inoculums filtered at 1–5  $\mu\text{m}$  and the infectious agent was not found to pass the 1  $\mu\text{m}$  filter. This may partially explain why on occasion batches of oysters from some hatcheries reared in 5  $\mu\text{m}$  filtered water developed and transmitted JOD when relocated and others from the same batch remained unaffected by the disease. In each case, results of laboratory experiments supported findings of field studies. Oysters produced typical conchiolinous shell lesions and mortalities comparable to those observed in natural infections. Saltwater aquarium medications used to treat infected oysters had differing affects on the disease progression. Maracyn (erythromycin), recommended for treatment of gram-positive bacterial infections, was the most effective medication to reduce mortality in JOD-infected oysters. Two effective ways to manage the effects of JOD is to spawn oysters earlier, allowing them to grow above 30 mm in size before disease onset, and development of disease resistant strains. One year old oysters, selected for survival of JOD and the presence of multiple severe shell checks (thickened ridges or growth rings believed to be evidence of surviving JOD infection), were spawned and reared under normal culture protocols by Frank M Flower and Sons, Inc. and the Cornell Marine Center. Offspring of these oysters survived JOD far better than susceptible oysters reared under the same conditions. Continued selective breeding of three generations of oysters showed no improvement in resistance. Growers using these spawning methods continue to have minimal losses to JOD.

**THE INCIDENCE OF JUVENILE OYSTER DISEASE AT FISHERS ISLAND OYSTER FARM, FISHERS ISLAND, NY.** Steve Malinowski, Fishers Island Oyster Farm, Box 402, Fishers Island, NY 06390

During 1988–1990, Fishers Island Oyster Farm attempted to integrate the culture of juvenile oysters, *Crassostrea virginica*, into an existing adult oyster growout system in West Harbor, Fishers



Island Sound, NY. This site (depth = 2–3 m at low water) is significantly influenced by Block Island Sound. Maximum surface water temperature is 22°C. The adult growout system consisted of an array of longlines used to suspend lantern nets. Nets were stocked with one year old oyster seed (size range approx. 30–50 mm) obtained from Ocean Pond, Corp., a producer of seed oysters utilizing a salt pond (salinity = 12–20 ppt) on Fishers Island, NY. Growout of this large seed to market size occurs in 6–18 months. In an effort to increase the quantity of large seed oysters available to stock this system, 1mm juveniles were cultured in a land based upwelling system, grown to a size of 4 mm, and stocked in pearl nets (34 × 34 cm) at densities ranging from 200–500 oysters per net. The pearl nets, in vertical arrays of 4 nets, were deployed at the adult growout site during July for 3 consecutive years. Each year, an initial period of satisfactory growth was followed by an episode of high mortality (50–95%) during August. Conchiolin deposition was noted in live and dead oysters all three years. After three years, culture of first year oysters was abandoned at this site. Since then, seed production at the salt pond, where JOD has never been observed, has been increased and provided 100% of the 30–50 mm seed required to stock a significantly expanded adult growout system in West Harbor.

**JOD AT OYSTER BAY, NEW YORK.** David Relyea, Frank M. Flower & Sons, Inc., P.O. Box 88, Oyster Bay, NY 11771

In July of 1990, Frank M. Flower and Sons (FMF) experienced it's first hatchery oyster seed mortalities from Juvenile Oyster Disease (JOD). At the time it was called "Unexplained Mortality". Samples were sent to NMFS, Rutgers and VIMS. No pathogen was identified. Heavy mortalities continued in '91, '92, and '93. During that time Frank M. Flower was learning how to cope with the problem as research progressed. By 1994, oyster seed production was back to normal levels due to strategies developed by FMF in conjunction with researchers. To date it is still questionable as to what actually causes JOD.

**FIELD TRANSMISSION OF JUVENILE OYSTER DISEASE ON LONG ISLAND AND FLOW/DENSITY/MORTALITY STUDIES.** Gregg Rivara, Cornell University Cooperative Extension of Suffolk County, Southold, NY 11971; Stan Czyzyk, Long Island Mariculture Technologies, Holbrook, NY 11741

Field transmission of JOD to the Suffolk County Marine Environmental Learning Center (SCMELC) occurred with transfer of broodstock and seed oysters in 1992 and 1993 from Long Island hatcheries. In 1994 a flow/density study was undertaken at the SCMELC with support from NOAA's Oyster Disease Research Program.

A three tank, 27 silo upflow nursery system was constructed. Each tank served as a flow treatment; low (4 L/min per silo), medium (20 L/min) and high (40 L/min). Within each tank three

target stocking densities were triplicated: low (one liter per silo), medium (six liters) and high (12 liters). Tanks were fed unfiltered creek water from a 3.4 m<sup>3</sup> head tank filled by two 2-horsepower centrifugal pumps.

Silo units were initially stocked on 11 July 1994. Shell height at this time averaged 8 mm and stocking densities were well below targets. Each week, for a period of nine weeks, the volume and mortality (number dead per 100 randomly-selected oysters) of each silo, along with shell height measurements (live and dead) were recorded. Physical parameters measured weekly included temperature, salinity, Secchi depth and a check of flow rates. As the actual volume exceeded the target volume for each silo, oysters were randomly removed to bring the volume back down to the treatment level.

Substantial mortalities (>20%) were seen by week three in low flow silos; by week four mortalities in these silos averaged 60%. At this time mortalities in high flow silos were at or under 33%, and remained at this level until the end of the experiment. Weekly volumetric increases were higher in medium and high flow silos as compared to low flow silos. Flow affected growth and survival more than stocking density.

It is not known to what degree nutritional stress caused by low flows and high densities affects the manifestation of JOD. It is that suggested that silos can be highly stocked without excessive mortalities as long as relatively high flow rates are maintained; at the SCMELC this equates to 0.03 liters of flow per square centimeter of silo screen. These results prompted the development of the axial flow floating upweller system at Cornell in 1995.

Anecdotally, the authors saw free-floating oysters in silos during these experiments and in years prior. Upon observation with a dissecting scope, these oysters were found to be alive. It is hypothesized that the cause of death from JOD is the self "shucking" of the oyster when at least one adductor muscle attachment site is severed by conchiolin deposition, causing the oyster to lose control of its gape and its ability to keep potential pathogens and predators out of the shell cavity.

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**JUVENILE OYSTER DISEASE (JOD) IN CRASSOSTREA VIRGINICA: SYNTHESIS OF KNOWLEDGE AND RECOMMENDATIONS.** Katherine Boettcher, 11208 Beechwood Pointe, Smithfield, VA 23430; Roxanna Smolowitz, Marine Biological Laboratory, 7 MBL St Woods Hole, MA 02536; Earl J. Lewis, NOAA NOS, 904 S. Morris St., Oxford, MD 21654; Bassem Allam, MSRC, Stony Brook University, Stony Brook, NY 11794; Harry Dickerson, College of Veterinary Medicine, University of Georgia, Athens, GA 30602; Susan Ford, Haskin Shellfish Laboratory, Rutgers University, P.O. Box 687, Port Norris, NJ 08349; Anwar Huq, Center of Marine Biotechnology, University of Maryland, Columbus Center, 701 E. Pratt St., Baltimore, MD 21202; Kimberly Reece, Virginia Institute of Marine Science,

P.O. Box 1346 Gloucester Point, VA 23062; **Gregg Rivara**, Cornell Cooperative Extension, 3690 Cedar Beach Rd., Southold, NY 11971 and **Cheryl M. Woodley**, NOAA NOS, 331 Ft. Johnson Rd. Charleston, SC 29412

The Juvenile Oyster Disease Workshop held in conjunction with the 26th Milford Aquaculture Seminar chronicled two decades of an industry's fight against juvenile oyster disease to save their businesses; their partnership with the research community to find the causative agent(s) for this devastating disease of cultured young oysters; and their experiences in learning how to manage this disease to keep an industry alive.

#### THE ISSUE

Juvenile oyster disease (JOD) is a significant disease of juvenile cultured oysters in the Northeastern United States. The earliest report dates back to the mid-1980's, however the peak of the devastation experienced by most nursery operations occurred in the 1990's. Those able to withstand the economic losses wrought by this disease did so by devising effective management methods and the disease no longer affects the viability of those commercial operations. Nevertheless, as new oyster aquaculture ventures emerge in the Northeast (driven in part by the need of fishing communities to retool after the devastating collapse in numbers of feral finfish and shellfish in the area), JOD outbreaks continue to appear sporadically in other culture sites previously thought to be unaffected or those only rarely affected. The annual variability in occurrence and intensity often results in unexpected and marked economic loss to individual producers. Basic and applied research conducted over the last 20 years has resulted in significant advances in our knowledge of the disease, its etiology, and methods for its mitigation; however, questions remain. A more thorough understanding of the disease pathogenesis, including host/pathogen interactions and other factors (i.e., anthropogenic or physical and/or biological agents in the environment) that contribute to JOD-epizootics, will facilitate the development of reliable surveillance measures for early warnings, help identify threat levels for a given location, and provide a basis for growers and regulators to make informed decisions.

#### THE DISEASE

JOD is generally observed after water temperatures have reached 20°C and at sites where salinities are above 18 ppt and primarily affects animals between 15–25 mm in shell height. The first clinical sign of the disease syndrome is decreased growth followed by the appearance of gross signs that include extreme cupping of the left valve, loss of the growing edge of the right valve resulting in uneven valve margins with retraction of the mantle edges within the shell (mantle recession) and deposition of abnormal brownish layers of conchiolin (organic shell matrix) on the inner surface of the affected shells. Mortality, which may reach 90%, begins within a week or two of the onset of slowed growth. Conchiolin deposition (a non-specific host response by oysters) is often observed in the form of a raised ridge around the retracted mantle edge. The conchiolin layer may also interfere with attachment of the adductor muscle. Histological lesions in the mantle occur before abnormal conchiolin is deposited. Depending on the

size of the animals, not all of the particular clinical signs may be present in an individual oyster (however they can be observed within a random sampling of a production lot). For example, oysters less than 15 mm shell height often die before they are able to mount the characteristic conchiolin response. Animals between 15 and 25 mm shell height typically show all gross signs of disease (including abnormal conchiolin deposition and uneven valve margins). In addition, the highest mortality rates occur among oysters that are less than 25 mm. Juveniles over 25 mm shell height often show abnormal conchiolin deposition, but growth effects (including uneven valve margins) and accompanying mortality are less common. Animals surviving JOD may develop external shell checks that result when normal growth is resumed.

Typical histological findings with JOD begin early in the disease process. Initially, there is increased hemocytic inflammation in the sinusoidal tissues of the mantle underlying the shell epithelium that is associated with attenuation and cuboidal metaplasia and/or hyperplasia of shell epithelium. In more advanced lesions, necrosis of shell epithelium occurs, resulting in ulceration and severe hemocytic inflammation. Other histological findings include diapediasis of hemocytes across the epithelium into the extra-pallial space (located between the epithelium and shell), accumulation of necrotic cellular debris and bacteria between layers of abnormal conchiolin in that space and occurrence of small coccoid bodies in the affected epithelium. Although the precise cause of death is unknown, these lesions may result in destruction of the adhesion between the shell and adductor muscle causing a critical loss of function. Secondary infections, loss of hemolymph and generalized physiological dysfunction also may contribute to death.

Advances in management practices that are effective in helping to control JOD include:

- Increasing water flow in nurseries, including the use of floating upweller systems and mesh sizes of 6 mm or greater in grow-out containers.
- Decreasing stock density.
- Selective breeding has resulted in some stocks of oysters that demonstrate enhanced tolerance to JOD.

#### THE AGENT

*No set of criteria can provide absolute proof of causation but that guideline can, and should, be used to weigh evidence (Sir Austin Hill, 1965)*

In the late 1800's Robert Koch recognized the need for defining a causal relationship between a microbe and a specific disease. His postulates have since been used to guide the collection of evidence to determine if a given organism is the cause of a disease with the proof lying in the concordance of the evidence. Ideally, one should show 1) the parasite occurs in every case of the disease and under circumstances that can account for the pathology and clinical course of the disease; 2) the parasite occurs in no other disease as a fortuitous and nonpathogenic parasite; and 3) after being isolated from the body and repeatedly grown in pure culture, the parasite can again induce the disease and be reisolated. These postulates illustrate the underlying principles:

- Demonstration of a specific association with disease serves to rule out a commensal relationship.
- Isolation in pure culture on laboratory medium proves the existence of an independent living organism and provides a pure inoculum.
- The ability to reproduce disease by exposure to the pure culture of the microorganism demonstrates pathogenicity.
- The ability to re-isolate the microorganism in culture from an experimentally infected host strengthens the case and demonstrates replication within the animal.
- The microbiological evidence should also be in agreement with the pathological and circumstantial data.

Based on these postulates and additional supporting evidence, *Roseovarius crassostreae*, has been established as the etiological agent of JOD. This novel species is a gram-negative bacterium within the *Roseobacter* clade of the marine Alphaproteobacteria, and is the numerically dominant bacterium isolated from JOD-affected oysters. Their relative abundance was confirmed using a culture-independent approach (i.e. sequence analysis of 16S rDNA libraries from affected oysters). The association is consistent regardless of time, location or size of the animals. Thus, *R. crassostreae* is the only organism that completely satisfies Koch's first postulate. In agreement with Koch's second postulate, *R. crassostreae* is not found associated with any other oyster disease and is not found in healthy individuals. (Interestingly, a close relative has been implicated in black-band disease of corals, but the bacterium has not been isolated in culture). Both mortalities and JOD-like conchiolin have been induced in healthy oysters under laboratory conditions when challenged by injection with *R. crassostreae*, and the bacteria were re-isolated from affected individuals. *R. crassostreae* has also been detected in oysters coincident with the earliest microscopic lesions and at least one week prior to the appearance of gross JOD-signs. The bacteria appear to use polar fimbriae to preferentially colonize the inner shell surface, including the conchiolin deposits. Consistent with previous histological studies, *R. crassostreae* do not appear to invade the oyster soft tissues.

The disease can be spread directly among animals, but factors influencing its emergence are not well understood. Minor genetic variations in the 16S–23S rDNA internal transcribed spacer (ITS) region sequences of *R. crassostreae* strains have been identified and used for epizootiological applications. The data indicate that widespread dispersal of genotypes occurs more quickly than the time required for even slight divergence in the ITS. Some data also suggests that most new cases of JOD arise from indigenous *R. crassostreae* and that prolonged use of shellfish culture sites may contribute to their enrichment in those sites. Some *R. crassostreae* introductions may have resulted from direct transfer of adults or seed oysters.

It is still possible that other bacteria and/or protists may play a role in predisposing oysters to JOD or in the disease process itself. For example, JOD frequently occurs soon after exposure of the seed to a phytoplankton or zooplankton bloom, which in turn may be followed by a bloom of *Vibrio* spp. Whereas no particular *Vibrio* species has ever been associated with JOD, increases in total *Vibrio* levels in the environment (and in the oysters themselves) have been associated with at least some JOD outbreaks.

Elevated *Vibrio* levels, which appear before JOD symptoms and persist as the disease develops, may also contribute to mortality. In a recent molecular study into a possible protistan involvement in JOD (using cloned 18S ribosomal RNA sequences), a ciliate, *Me-sanophrys magna*, was identified in a population of JOD-affected animals. Subsequent PCR-based screening of those same oysters with primers specific for *M. magna* and *R. crassostreae* showed highly significant correlations with the presence of these two microbes in diseased tissues.

## CONCLUSIONS

After reviewing the history and findings presented during this workshop the invited external panel and members of the workshop recognize that significant advances in understanding the etiology of JOD have occurred. Nevertheless, they emphasize that many questions remain unanswered. They were asked to 1) identify salient gaps in our understanding of this disease and its process that will improve the management and control of JOD and 2) make recommendations as to the strategic research needs. The recommendations they put forth are as follows:

- Characterization of the environmental, physical and biological conditions that drive the disease process (e.g., salinity, temperature, and the potential involvement of algal blooms) will provide information for early warning and devising better management strategies.
- Development of additional (e.g., quantitative) diagnostics for early detection and surveillance of potential areas which may be at risk are needed to limit the spread of the disease.
- Elucidation of the pathogenesis of JOD is needed and includes determining the conditions that facilitate colonization of hosts by *R. crassostreae*, the minimum infectious dose, the identification and regulation of virulence factors, and a more thorough examination of the host responses to the infection.
- Identification of factors associated with oyster development and their role in susceptibility to JOD may provide insight into the restricted period during which the animals are susceptible.
- Understanding defense mechanisms (host response) of infected oysters is needed for insight into the biological basis of resistance.
- The ecology of *R. crassostreae* needs to be further investigated (e.g., factors influencing its distribution and abundance, reservoirs, and possible intermediate hosts).
- Further evaluation is needed of *Stappia stellulata* as a potential bacterial probiotic to prevent *R. crassostreae* colonization of oyster seed and subsequent mortalities.
- Future efforts directed at development of additional disease-resistant strains using information from research as well as survivor breeding will provide excellent management methods for this disease.

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GENETIC DIFFERENTIATION AMONG FOUR *CRASSOSTREA ARIAKENSIS* POPULATIONS IN ASIA BY MICROSATELLITE POLYMORPHISM

**TWO-YEAR PERFORMANCE OF TWO DISEASE-TOLERANT OYSTER STRAINS IN THE PATUXENT RIVER, MARYLAND.** George R. Abhe<sup>1</sup>, Carol B. McColough<sup>2</sup>, and Christopher F. Dungan<sup>2</sup>. <sup>1</sup>Morgan State University, <sup>2</sup>Maryland Department of Natural Resources.

Extreme mortalities among a standard strain of specific-pathogen-free (SPF) *C. virginica* at three sites in the Patuxent River, Maryland during the 2000–02 drought, led to our search for improved performances from available disease resistant native oyster strains. SPF spat of disease-tolerant CROSBreed and DEBY strains, alongside standard-strain oysters, were set and deployed in September 2003, at our same experimental sites. Mean station salinities ranged from 12.1–16.2 ppt during 2000–02, but have been generally lower since then, although salinity increases were observed during much of 2005. Mean incremental two-year growth was better for all strains combined at the mid- (62.8 mm) and down-river (61.5 mm) sites than upriver (49.4 mm), probably because of occasional marginal salinities at the upriver site during year 1. Two-year growth of DEBYs (64.5 mm) was better than CROSBreeds (56.5 mm) or standards (52.5 mm) based on net shell-length increases. After two years, prevalence and body burdens of *Perkinsus marinus* in experimental oysters are increasing, and light mortalities are beginning to occur, all of which are probably related to increasing salinity and increasing infection levels in feral host populations in the deployment areas. Whether disease levels and mortalities of experimental oysters remain at acceptable levels will depend on future salinities; whether DEBYs or CROSBreeds outperform standards will depend on future salinities and also on functional genetic effects. Differences should be apparent by the end of the third year.

**OYSTER (*CRASSOSTREA VIRGINICA*) REEF RESTORATION IN THE BELLAMY RIVER, NH: INITIATION OF A MULTI-SPECIES PROJECT.** Holly Abeels, Ray Grizzle, Jennifer Greene, and Krystin Ward. Jackson Estuarine Laboratory, University of New Hampshire.

The eastern oyster (*Crassostrea virginica*) plays an important ecological role in the Great Bay estuarine system, where the populations have declined due to disease and other factors. In 2000, the New Hampshire Estuaries Project (NHEP) developed a management plan with a goal of increasing oyster populations by adding twenty acres of 'restored' oyster bottom by the year 2010. The current project was developed in collaboration with NHEP and the City of Dover, NH to contribute to this goal and to begin multi-species habitat restoration in the Bellamy River estuary. The river bed was surveyed in fall 2004 using a towed video and DGPS system and bottom habitats classified to determine the extent of hard bottom available for planting of 'seed' oysters on cultch. Ten million larvae spawned from Great Bay broodstock were set onto shell cultch in two 3,000-gallon fiberglass tanks located at Jackson Estuarine Laboratory in July 2005. Once settlement had occurred,

the spat were transferred to a nursery raft located in an adjacent cove. After 3 months, approximately 400,000 spat (~40 mm shell height) had survived and were transferred to the study site where twelve mini-reefs were created in a 6000-m<sup>2</sup> area and one reef created in a 200-m<sup>2</sup> area. This area is located adjacent to an ongoing eelgrass restoration project, which will allow a multi-species community to develop. Future work will include sampling with towed video, counts and measurements of live oysters, and set out of mussel recruitment devices for transfer to multi-species habitat.

**THE ECONOMIC IMPLICATIONS ASSOCIATED WITH BIOFOULING IN SHELLFISH AQUACULTURE.** Charles M. Adams<sup>1</sup>, Sandra E. Shumway<sup>2</sup>, Robert B. Whitlatch<sup>2</sup>, and T. Getchis<sup>3</sup>. <sup>1</sup>Florida Sea Grant Extension Program, University of Florida, P.O. Box 110240, Gainesville, FL 32611, <sup>2</sup>Department of Marine Sciences, University of Connecticut, Groton, CT 06340, <sup>3</sup>Connecticut Sea Grant College Program, Groton, CT 06340.

Biofouling is an important issue to consider when designing a marine shellfish culture system. The costs associated with controlling biofouling in marine shellfish culture systems can be significant. This is particularly true for those systems which utilize the water column for the production process, such as rope, cage, and suspended net systems. This study examined several of the economic implications associated with bio-fouling in marine shellfish culture systems in the northeast U.S. region. A survey was sent to commercial marine shellfish aquaculturists within the region. These operations predominantly culture clams, oysters, and mussels. They utilize a wide variety of culture methods. Each survey recipient was asked to consider the economic implications of bio-fouling on their culture system, as well as their product marketing efforts. Survey recipients were asked to describe how biofouling affected their production, including impacts on growth rate, harvest size, yield, etc. Information on current methods of control was also solicited. Survey recipients were also asked to provide an estimate of the annual costs associated with bio-fouling control. Similar questions were posed regarding the impact that bio-fouling has on the marketability of the harvested product. The responses provide insight into the differential effects that biofouling has on commercial shellfish culture operations, when considering species cultured, production method utilized, culture system location, targeted market, and other factors. This information will be useful when considering the efficacy of future innovative methods for controlling biofouling in shellfish culture operations within the northeast and other regions of the U.S.

**OYSTER RECRUITMENT AND EARLY GROWTH PATTERNS IN THE BARATARIA ESTUARY.** Cassandra D. Addison and Earl J. Melancon, Department of Biological Sciences, Nicholls State University, P.O. Box 1, Thibodaux, LA 70310.

We documented temporal and spatial spawning, recruitment, and early growth of oysters within the historically productive zone of the Barataria Estuary from April 2004 to November 2005. The

study area falls within the outfall of the Davis Pond Mississippi River Diversion, which has flowed minimally with no measurable impact to oysters. Hence, data reflect responses to natural conditions, especially the synergism of salinity and temperature. During the study there were two reduced-salinity events ( $<5$  ppt), accompanied by two tropical storms and three hurricanes. A spawn and recruitment failure occurred throughout the estuary in May/June 2004 due to low salinities ( $<5$  ppt). Oysters reabsorbed gonad tissue and died by mid-summer, except at the most down-estuary site. The May 2005 spawn proceeded normally, but recruitment failed. Recruitment failure may be due to a lack of food availability. Fortunately, successful fall spawn and recruitment events occurred in 2004 and 2005. Preliminary findings suggest a spatial significance in recruitment abundance, with down-estuary areas best. Individual fall 2004 spat were tagged and placed in cages at two sites. In 11 months, from October 2004 to August 2005, the down-estuary site produced oysters with a mean length of  $62.7 \pm 7.5$  mm, while up-estuary oysters grew to  $47.5 \pm 7.9$  mm. Fall-recruited spat can grow to a desired commercial-sized seed in one year, with the more down-estuary site showing faster growth. This information becomes crucial for natural resource managers and oystermen, especially during low-salinity conditions. Adaptive up-estuary to down-estuary management strategies for seed can be developed to allow diversions and oysters to co-exist.

**GROWTH AND GONAD PRODUCTION OF THE SEA URCHINS *HEMICENTROTUS PULCHERRIMUS* AND *ANTHOCIDARIS CRASSISPINA* AMONG ALGAL SERE.** Yukio Agatsuma<sup>1</sup>, Hisayuki Arakawa<sup>2</sup> and Kazuya Taniguchi<sup>1</sup>, <sup>1</sup>Graduate School of Agricultural Science, Tohoku University, <sup>2</sup>Tokyo University of Marine Science and Technology.

To clarify the differences in the growth and gonad size of the sea urchins *Hemicentrotus pulcherrimus* and *Anthocidaris crassispina* among algal sere, the study was conducted in July 2002 at a fucoid bed and a kelp bed in Gobo and two algal turfs in Mihama, Wakayama Prefecture in southwestern Japan. The growth of *H. pulcherrimus* in the fucoid bed and the kelp bed, where the large perennial *Myagropsis myagroides* and *Ecklonia cava* at the climax stage dominated, respectively, are higher than that in the algal turfs dominated by the small perennial articulated corallines at the seral stage. While the most rapid growth of *A. crassispina* was observed in the fucoid bed, the gonad indices (gonad wet weight x100/body wet weight) of both species of the sea urchins were highest in the fucoid bed. These results suggest that growth and gonad production of *H. pulcherrimus* and *A. crassispina* are affected by algal sere as reported in *H. pulcherrimus* and *Strongylocentrotus nudus* in northern Japan, although they differed among dominant species of algae at the climax stage.

**ASSESSMENT OF THE ABILITY OF *CRASSOSTREA ARIAKENSIS* HEMOCYTES TO KILL *PERKINSUS MARINUS* IN VITRO.** Mohammad R. Alavi, Jose A. F. Robledo, Eric J. Schott, Keiko Saito, Satoshi Tasumi, and Gerardo R. Vasta, Center of Marine Biotechnology, UMBI, University of Maryland, 701 E. Pratt St. Suite 236, Baltimore, MD.

The eastern oyster, *Crassostrea virginica*, has been reduced to such low numbers in the Chesapeake Bay that the introduction of the Asian oyster *C. ariakensis* is being considered by the industry, and state and federal agencies. To assess the susceptibility of *C. ariakensis* to *Perkinsus marinus* (Dermo) we examined the capacity of *C. ariakensis* hemocytes or plasma to kill or inhibit proliferation of *P. marinus* trophozoites, and compared it to that of *C. virginica*. *P. marinus* trophozoites were exposed *in vitro* to oyster hemocytes or plasma (at six infection ratios) for 24 and 72 hrs and the parasite's ability to proliferate after the exposure was assessed by culture in nutrient medium for four weeks, with weekly measurements of cell densities. Although both *C. virginica* and *C. ariakensis* hemocytes apparently reduced the viability of most of the exposed parasites, even at the ratio of 16:1 (hemocytes: parasites) a small proportion of the parasite survived and proliferated upon addition of culture medium. To rigorously discriminate adherent trophozoites from those phagocytosed by the oyster hemocytes, we developed a method based on a chloromethylbenz-amido derivative of a dialkylcarbocyanine dye (CM-DiI), a lipophilic molecule that can remain highly fluorescent when incorporated into cell wall and can be quenched by trypan blue if the parasite remain extracellular. Using this method, both degraded and intact parasite could be detected within hemocytes 24 hrs post-exposure, confirming our previous results. (Supported by grants from the Maryland DNR and NOAA).

**EFFECTS OF HARMFUL ALGAE ON OYSTER GROWTH.** J. Alexander<sup>1</sup>, D. Meritt<sup>1</sup>, A. Pareletti<sup>1</sup>, S. Alexander<sup>1</sup>, D. Stoecker<sup>1</sup> and P. Glibert. <sup>1</sup>University of Maryland Center for Environmental Science.

Introduction of the Asian oyster, *Crassostrea ariakensis*, to Chesapeake Bay is of considerable interest in the broader plans for Bay restoration. Overfishing, loss of habitat, and disease have caused dramatic losses in the native oyster, *Crassostrea virginica*, and it is thought that the Asian oyster may succeed where the native oyster has not because of its high growth rates, among other factors. One of the manifestations of poor water quality in Chesapeake Bay is the development of harmful algal blooms. Critical to an understanding of the potential success of the Asian oyster in Chesapeake Bay is knowledge of the impact of common harmful algae on oyster growth. The first phase of this investigation included a comparison of the effect of harmful algae on spat growth, measured as shell growth, of both *C. virginica* and *C. ariakensis*. Effects of exposure to *Prorocentrum minimum* were compared to that of *Isochrysis galbana*, one of the standard hatchery feed or-



ganisms, and a non-fed control treatment. Valve height of cultch-less test oysters, prepared by grinding away a portion of the shell periphery, following guidelines from the EPA Oyster Acute Toxicity Test, was measured before and after 96 hrs exposure to each treatment. Feces, pseudofeces, and gut contents were also collected from sample animals to compare digestion. Initial results indicate that *C. virginica* was impacted negatively by *P. minimum* to a greater extent than was *C. ariakensis*.

**IN VITRO INVESTIGATIONS OF QUAHOG PARASITE UNKNOWN (QPX).** Bassem Allam, Deenie M. Buggé and Mickael Perrigault. Marine Sciences Research Center, Stony Brook University, Stony Brook NY 11794–5000.

Quahog parasite unknown (QPX) has been successfully isolated from clams from different geographic locations in the Northeast. This study uses an *in vitro* approach to investigate the virulence and specificity of different QPX isolates. A fluorometric growth measurement technique and a neutral red cytotoxicity assay were used to investigate interactions between QPX and components from different bivalve species and strains. Our experiments demonstrate that QPX produces extracellular virulence factors that are cytotoxic to *M. mercenaria* hemocytes. This cytotoxicity may play an important role in supporting QPX infection and proliferation within the host. Experiments also indicate that QPX growth in *M. mercenaria* is tissue-specific and that some tissues possess anti-QPX properties. For example, host foot tissue supports *in vitro* QPX growth while growth in mantle or gill tissue is strongly inhibited. Bivalve plasma also contains anti-QPX factors which may play a role in host defense. Plasma from species not known to develop QPX disease contains higher anti-QPX activity than *M. mercenaria* plasma. The *in vitro* inhibitory effect of plasma seems however to be time-dependent and lasts up to two days since further incubation results in an enhancement of QPX growth. These and future *in vitro* investigations will increase our understanding of QPX infection and disease development. For instance, studies targeting tissues where anti-QPX activities are localized may provide a good strategy for the determination of biological bases of QPX resistance in clams.

**RISKY BUSINESS: PROSPECTIVE LOOK AT TRIPLOID *C. ARIAKENSIS* AQUACULTURE.** Standish K. Allen Jr., Virginia Institute of Marine Science, P.O. Box 1346, Great Rd., Gloucester Point, VA 23062.

Among the options under consideration by the current Environmental Impact Statement (EIS) process on the introduction of *C. ariakensis* to Chesapeake Bay is that of aquaculture of triploid *C. ariakensis*. The premise for this option is that sterile triploids can eliminate or lessen the risk of inadvertent introduction. Triploidy is produced by crossing tetraploid with diploid brood stock, yielding high proportions of triploids—but not 100%. Our experience

with certifying eight batches (average sample size for each certification: 3558) of triploids produced in this way indicates that, on average, batches are  $99.86 \pm 0.094\%$  (95% CI) triploid. Essentially, the risks posed by triploid aquaculture boils down to the risk of reproduction from the one in 1000 diploids scattered among the crop. We have overseen two major industry aquaculture trials with triploid *C. ariakensis*. Because of Federal permitting requirements by the Army Corps of Engineers for these trials, various other Federal agencies, as well as States and NGOs, have weighed in on provisions for permitting. Risk has been quantified by a simple probability/ demographic model to estimate the number of surviving spat that result from various deployment options. Moreover, these risk calculations have been deemed cumulative, such that those risks posed in a previous industry trial with triploids are summed with those for subsequent ones. Clearly, this approach leads to spiraling impossibility to continue these trials, and seemingly, excludes the opportunity for commercial scale aquaculture. A more “liberal” approach to engaging triploid aquaculture and dealing with its concomitant risks will be discussed.

**SHORT-TERM EVALUATION OF MORTALITY FROM BONAMIA SP. INFECTIONS IN *CRASSOSTREA ARIAKENSIS* AT TWO LOCATIONS IN SOUTHEASTERN NORTH.** Troy Alphin<sup>1</sup>, Ami Wilbur<sup>1</sup>, Ryan B. Carnegie<sup>2</sup> and Martin H. Posey<sup>3</sup>. <sup>1</sup>UNC-Center for Marine Science, 5600 Marvin K. Moss Ln, Wilmington, NC 28909, <sup>2</sup>Virginia Institute of Marine Science, Gloucester Point, VA, <sup>3</sup>Department of Biology and Marine Biology, UNC-Wilmington.

The Suminoe oyster, *Crassostrea ariakensis*, has been proposed as a potential aquaculture species for several states along the Atlantic coast of the United States, including Maryland, Virginia, and North Carolina. A 2003 trial of *C. ariakensis* in Bogue Sound, North Carolina, resulted in high mortality due to infection by *Bonamia* sp. The presence of *Bonamia* sp. may represent a significant impediment to the future culture of *C. ariakensis* along the east coast. As part of a field study evaluating the presence of *Bonamia* sp. in coastal North Carolina, *Ostreola equestris*, a species that may serve as a reservoir host, was sampled from a variety of locations from Bogue Sound to Wilmington. *Bonamia* sp. was detected in the Wilmington area in July 2005 and juvenile triploid *C. ariakensis* were deployed at two Wilmington locations in late September to determine if parasite transmission to this non-native species would occur. The first site was a research bottom lease, at the mouth of Hewletts Creek, NC and the location where *Bonamia* sp. was observed in *O. equestris*, while the second site was located 3 km south along the ICW at a site where *O. equestris* had not been noted. *C. ariakensis* were deployed for eight weeks with weekly monitoring of *Bonamia* sp. prevalence and mortality, and biweekly monitoring of growth. *Bonamia* sp. infections in *C. ariakensis* were noted at both locations, with a higher *Bonamia* sp. prevalence

at the research lease site. Peak mortality approached 40% during the 5th week of the study.

**IN VITRO PROTEASE PRODUCTION BY QPX.** Robert S. Anderson, Tracey M. Luskey and Maureen A. Strauss. University of Maryland Center for Environmental Science, Chesapeake Biological Laboratory, P.O. Box 38, Solomons, MD 20688.

QPX secretes virulence factors, including proteases that contribute to success in its host. *In vitro* production of QPX proteases was followed by zymography for four to six weeks, using trisglycine gels with 0.1% gelatin incorporated as a substrate. Little or no protease activity was detected in unsupplemented Kleinschuster's medium, in which QPX was cultured at 11°, 20° or 27° C. However at 20° C, supplementation with fetal bovine serum (FBS) resulted in the production of 6–7 QPX-dependent proteases, plus 4–5 FBS-associated proteases. After six weeks of incubation, QPX proteases included a >200 kD band plus bands at ~70, 51 and 46 kD; other proteases were weakly or occasionally expressed. The 46 kD band was consistently the major QPX protease. Expression of QPX 46 kD protease showed temperature dependency: it was maximally (+++) expressed by one week at 27° C, but seen as a faint (+/-) band after three weeks at 11° and 20° C. Experiments were carried out to determine if the presence of host tissue extracts in the medium would modify the expression profile of QPX proteases. FBS and homogenate proteases interacted to produce strong bands of activity that made observation of QPX proteases impossible. Therefore, homogenate effects were measured in FBS-free medium. Unique, active (++) ~60 & 65 kD proteases were seen throughout the six week course of the homogenate-QPX study. The major (46 kD) protease in FBS-supplemented cultures was weakly expressed, and only after three weeks. These results suggest that the microenvironment of QPX may influence its pathogenicity.

**RETURNING TO SALTWATER POND CULTURE OF CRASSOSTREA VIRGINICA.** William D. Anderson<sup>1</sup>, William A. Cox<sup>2</sup> and Jack M. Whetstone<sup>3</sup>. <sup>1</sup>South Carolina Department of Natural Resources, P.O. Box 12559, Charleston, SC 29422, <sup>2</sup>Island Fresh Seafood, Inc., <sup>3</sup>Clemson University.

Using saltwater ponds to cultivate oysters can be traced back to the Romans in the first century B.C., and even earlier to Chinese aquaculture. In South Carolina and other east coast states, growing large, single eastern oysters in saltwater ponds and abandoned impoundments has been practiced since the 1800s. South Carolina's renowned "mill pond oyster" floated as spat on logs into saw-mill ponds, grew near the surface, dislodged, and if it survived in the mud bottom environment, matured into an epicurean delight. Oyster culturists observed pond oysters' adductor muscles to be at least one-third larger than their estuarine siblings, ostensibly to avoid smothering by siltation on the mud bottom.

Saltwater ponds generate a favorable environment for controlled autotrophic production and can sustain a higher instantaneous biomass throughout the year compared to adjacent, productive estuarine waters. Through intake filtration, circulation adjustments, salinity and water level control, ponds can be intensely managed to inhibit parasites, predators and disease while accelerating growth.

Optimum conditions for pond and estuarine aquaculture of eastern oysters have evolved recently in SC due to a successful clam mariculture infrastructure seeking diversification, strong market demand for single local oysters, hatchery technology advances and the capacity to cultivate large (>57 mm) juvenile oysters in a controlled environment.

Island Fresh Seafood, a South Carolina Corporation has spawned *C. virginica* seed that has grown 63 mm in six months, considerably faster than wildstock oysters. This presentation discusses salt water pond characteristics, production of seed oysters and South Carolina's cooperative fisheries research initiatives to engender single oyster aquaculture by commercial shellfishermen.

**AN OVERVIEW OF BIVALVE POPULATION RESTORATION EFFORTS IN FLORIDA WATERS.** William S. Arnold. Florida FWCC Fish and Wildlife Research Institute, 100 8th Ave. S.E., St. Petersburg, FL 33701.

The coastal zone in Florida is being developed at a rapid pace, placing increasing pressure on nearshore marine resources including bivalve molluscs such as scallops, clams, and oysters. In an effort to mitigate the impacts of coastal development and to maintain viable and broadly distributed populations of these species, a variety of approaches have been applied to rebuild or reestablish populations. These approaches include transplanting adults, planting seed, releasing larval or post-larval animals, and constructing habitat. Success has varied considerably depending upon the method employed, the target species, and the interactive effects of species and habitat. Among many lessons that have been learned, one of the most essential is that the cause of collapse must be identified and either ameliorated or circumvented prior to initiating a restoration effort. Otherwise, the likelihood of success is low. Because similar development pressures are either underway or imminent throughout the United States, the lessons learned in Florida should be of interest to a broad array of coastal zone scientists and managers.

**DOES DISTANCE FROM SUBTIDAL CHANNEL INFLUENCE USAGE OF NEWLY ESTABLISHED INTERTIDAL OYSTER REEFS BY TRANSIENT AND RESIDENT FAUNA.** Stephen J. Artabane, Martin H. Posey and Troy D. Alphin. University of North Carolina, Department of Biology and Marine Biology, 601 S. College Rd., Wilmington, NC 28903.

The habitat value of oyster reefs (*Crassostrea virginica*) to many finfish and decapods may vary significantly with landscape factors such as proximity of adjacent habitats, distance to source

areas, and reef architecture. We examined how distance of intertidal reefs from subtidal channels and low tide refuges for transient fauna influences reef use by both resident and transient fauna. Four transects of artificial reefs were created using clean oyster shell and placed at distances of 5 m, 30 m, and 55 m from a subtidal channel on a tidal flat in southeastern North Carolina. Transient finfish and crustaceans were sampled during flood tide using Breder traps and surround nets. Resident species were sampled using shell excavations and sediment cores. Preliminary data suggest preferential use of reefs over non-reef controls and use of edge habitat over interior habitat for the dominant transient species: pinfish, spot and mummichog. Conflicting patterns were observed between both sampling methods for pinfish, with Breder trap data indicating preference for reefs furthest from channel and surround net data indicating preference for reefs closest to the channel. No clear pattern of use was observed for mummichog and juvenile spot. Excavations show higher settlement of oyster spat on reefs located at 5 m from the subtidal channel, but no differences among distances for recruitment of mud crabs, *Panopeus herbstii*. Measurements of predation using tethered grass shrimp, *Palaemonetes pugio*, and outplanted hard clams, *Mercuraria mercenaria*, were conducted to measure actual reef utilization but results were inconclusive.

**ENSO AND CRUSTACEAN POPULATIONS OF PERÚ: ARE THE LANDINGS RECORDS RELATED TO THE SEA SURFACE TEMPERATURE?** Cynthia M. Asorey<sup>1</sup>, Javier Calcagno<sup>1</sup> and Gustavo A. Lovrich<sup>2</sup>, Facultad de Ciencias Exactas y naturales-UBA/CENSOR EU, <sup>2</sup>Centro Austral de investigaciones CADIC, Ushuaia/CENSOR EU.

The marine ecosystem of the south eastern Pacific is made up of a particular system of superficial currents that make the coasts of Chile and Peru some of the world's most productive fishing zones. Few studies have been conducted to examine the population dynamics of commercial crustaceans and ENSO. This concern is probably due to the low economic value of these species, when compared to other commercial ones. In this work, mean and maximum temperatures registered at the Chicama station (07° 41' S, 79° 26' W), between 1970 and 1999 were correlated with the crustacean landing records of the Instituto del Mar del Peru. We found a positive correlation between the total crustacean landings of shrimp species (*Litopenaeus spp.*, *Farfantepenaeus californiensis* and *Xylophoropeneae riveti*) and both the annual mean temperatures ( $r = 0.43$ ,  $p < 0.05$ ) and the maximum temperatures ( $r = 0.39$ ,  $P < 0.05$ ). We also found a negative but not significant, correlation ( $r = -0.22$  for the mean SST and  $r = -0.28$  for the maximum SST,  $p > 0.05$ ), for the crab species (*Cancer sp.*, *Platyxanthus sp.*). These results coincide with earlier works that report on the increase of mortality rates and migrations to deeper water of crab species. Coincidentally, during the ENSO, commercial shrimps

move south from off Ecuador to off Perú, where fishers adapt their fishing arts to catch shrimp.

**INFLUENCE OF TEMPERATURE ON THE METABOLISM OF *MUNIDA GREGARIA* (= *M. SUBRUGOSA*) (CRUSTACEA: ANOMURA).** M. C. Avalos<sup>1</sup>, O. Heilmayer<sup>2</sup> and K. Paschke<sup>3</sup>, <sup>1</sup>CADIC (Centro Austral de Investigaciones Científicas), Ushuaia, <sup>2</sup>Alfred Wegener Institute for Polar and Marine Research, Brem., <sup>3</sup>Instituto de Acuicultura—Universidad Austral de Chile, Pue.

*Munida gregaria* is an epibenthic galatheid crab of 5 to 7 cm total length that has a key role in the coastal subantarctic ecosystem of South America. Although plenty of information on the species related to growth, reproduction, abundance and distribution is available not much is known about physiological adaptations to subantarctic temperatures. This preliminary study aimed to determine standard and active metabolism of *M. gregaria* acclimated at three different temperatures. Animals were caught off Puerto Montt (47°S), Chile, with an underwater vacuum at ~10 m depth. Oxygen consumption of animals ( $n = 6$  per treatment; mean carapace length =  $13.5 \text{ mm} \pm 1 \text{ mm}$ ) acclimated to 6°, 12° and 18°C was measured. A closed system was used for respiration measurement. Oxygen content was determined with microoptodes connected to a single channel MICROX TX3 array. After standard metabolism (defined as oxygen consumption of unfed, unstressed animals) was measured, animals were forced to move and be active. Oxygen consumption was measured to obtain active metabolism. Results show that standard metabolism of *M. gregaria* was significantly positive affected by temperature (6°C =  $0.105 \text{ mgO}_2 \cdot \text{h}^{-1} \cdot \text{g}^{-1}$ ; 12°C =  $0.161 \text{ mgO}_2 \cdot \text{h}^{-1} \cdot \text{g}^{-1}$ ; 18°C =  $0.232 \text{ mgO}_2 \cdot \text{h}^{-1} \cdot \text{g}^{-1}$ ). Active metabolism, however, could only be measured in few animals. In the cases the animals showed high levels of activity, a significant non-temperature dependent 2 to 3 fold increases in their oxygen consumption was measured. Our data provide preliminary insight into the physiology of this key species and help setting future experiments on this species, in order to study its thermo-tolerance in a latitudinal temperature context.

**INFLUENCE OF SALINITY ON THE DISTRIBUTION AND ABUNDANCE OF LARVAE OF DOMINANT OYSTER-REEF DECAPODS IN SOUTHWEST FLORIDA.** Bethany M. Bachelor<sup>1</sup>, S. Gregory Tolley<sup>1</sup> and Scott E. Burghart<sup>2</sup>, <sup>1</sup>Florida Gulf Coast University, Coastal Watershed Institute, 10501 FGCU Blvd S., Ft. Myers, FL 3396, <sup>2</sup>University of South Florida, College of Marine Science.

Spatial and temporal variability in recruitment of associated organisms to oyster reefs is dependent upon a number of factors including larval supply, which, in turn, is influenced by freshwater

inflow and salinity. Distribution and abundance of larvae of the three dominant decapod crustaceans found on oyster reefs in Estero Bay, Florida (*Petrolisthes armatus*, *Eurypanopeus depressus* and *Rhithropanopeus harrisii*) were quantified using monthly plankton tows. Sampling included stations near passes connecting to the Gulf of Mexico, in the open waters of the bay, and in association with tidal tributaries. Larval densities of the marine stenohaline *P. armatus* were greater in bay waters and near passes. Larvae of the euryhaline *E. depressus* occurred abundantly throughout the bay and in association with tidal tributaries. Larvae of *R. harrisii*, a species known to favor reduced salinities, were most abundant near tidal tributaries. Densities of *P. armatus* and *E. depressus* peaked at the end of the dry season (May) and were depressed with the onset of seasonal rains (June). In contrast, *R. harrisii* larvae were in short supply during dry months but were abundant during the wet season. Weighted mean salinity of capture was higher for *P. armatus* (29.72) and *E. depressus* (29.88) than for *R. harrisii* (6.72), and larval density was positively correlated with salinity of capture for *P. armatus* and *E. depressus* and negatively correlated for *R. harrisii*. These results suggest the importance of considering the effects of salinity on spatial and seasonal variation in larval supply in understanding recruitment dynamics of oyster-reef associates.

**LIFE HISTORY CHARACTERISTICS OF THE NONNATIVE GREEN MUSSEL (*PERNA VIRIDIS*), IN TAMPA BAY, FLORIDA.** Shirley Baker<sup>1</sup>, Bruce Barber<sup>2</sup>, Jon Fajans<sup>3</sup> and Patrick Baker<sup>1</sup>. <sup>1</sup>University of Florida, Department of Fisheries and Aquatic Sciences, 7922 N.W. 71st St., Gainesville, FL 32611, <sup>2</sup>Eckerd College, <sup>3</sup>Keys Marine Laboratory.

The green mussel, *Perna viridis*, has been intensively harvested and cultured for human consumption throughout its native range and has been transplanted outside its range numerous times for aquaculture. In August 1999, *P. viridis* was identified in Tampa Bay, Florida, the first report from North America. The overall objectives of our three-year research program were to monitor and predict the spread of *P. viridis* and major interactions with native bivalve species and phytoplankton communities. Here we examine the reproduction, recruitment, growth, and survival of *P. viridis*. Monthly samples were examined histologically for reproductive status and settlement plates were used to examine recruitment. Monthly quantitative samples were collected to estimate size distribution, cohort growth and survival. Based on both histological and recruitment data, *P. viridis* in Florida, as in Asia, has a primary spawning peak in spring and a lesser peak in autumn. Patterns were strongest in peak habitat (based on *P. viridis* density); the autumn peak timing or intensity varied in areas of lower *P. viridis* density. Growth rate was rapid—about 9 mm per month—and within the

range of growth rates reported in native habitats. Based on size, some *P. viridis* may become sexually mature and attain fishery size (~70–80 mm) within one year. As demonstrated in this study, green mussels successfully reproduce in Florida, resulting in dense populations throughout Tampa Bay.

**RESTORATION OF INTERTIDAL OYSTER REEFS AFFECTED BY INTENSE BOATING ACTIVITY IN SHALLOW FLORIDA WATERWAYS.** Andrea Barber<sup>1</sup>, Linda Walters<sup>1</sup> and Anne Birch<sup>2</sup>. <sup>1</sup>University of Central Florida, <sup>2</sup>The Nature Conservancy.

In recent years, intertidal reefs of the oyster *Crassostrea virginica* in central Florida's coastal areas have suffered extensive losses due to wakes from recreational boats. The creation and enforcement of "no wake" zones in the area are unlikely. Thus there is an urgent need for an alternative restoration strategy before oyster reefs decline past the point of no return. The goal of this project is to implement a scientifically-based restoration technique that minimizes wake damage from recreational vessels on intertidal reefs in Canaveral National Seashore (CANA). To accomplish this, we will test a range of restoration measures to determine the optimal design that best increases: 1) the numbers of oysters, 2) 3-D structure of our intertidal reefs, and 3) biodiversity and abundances of sessile and motile species on reefs. These restoration measures will include all combinations of leveling piles of disarticulated shells on reefs, placing seagrass seaward of reefs, and deploying miniature, mobile oyster reefs (restoration mats) to provide substrate for oyster recruitment and survival. Our restoration mat design includes affixing 36 drilled oyster shells to 0.4 × 0.4 m<sup>2</sup> pieces of black mesh. After completing this experimental objective, the majority of our effort will be to implement our optimal design to increase reef dimensions to historical levels within the bounds of CANA. Our goal is to restore 15–20% of the 400 reefs (60–80 reefs) in the project area (approximately 40 acres) that have been damaged by wakes from recreational vessels.

**THE NEW ZEALAND SEA URCHIN (*EVECHINUS CHLOROTICUS*) FISHERY.** M. F. Barker, Department of Marine Science, University of Otago, Box 56, Dunedin, New Zealand.

*Evechinus chloroticus* (Valenciennes) known locally as "kina" is a ubiquitous echinometrid sea urchin, endemic to New Zealand. It is one of the largest sea urchins known, with a maximum test diameter (TD) of 16–17 cm and is widely distributed around the main islands of New Zealand but is also found at the Chatham Islands to the west, and the Snares Islands to the south. It is generally found on rocky reefs in water less than 12 to 14 meters

deep although intertidal populations also occur, mainly in the north of the North Island. Adult urchins are normally found in areas with moderate currents and wave action, and are seldom found in areas of extreme exposure such as the surf shores that occur along much of New Zealand's west coast.

It was harvested by Maori people before the arrival of Europeans in New Zealand and was first harvested commercially in about 1983. In 2002 it was included in the New Zealand Quota Management System, of 10 regions around the country used to manage all of New Zealand's larger commercial fisheries. At the present time fisheries are supplying the domestic market almost exclusively because the poor taste and colour of the roe has resulted in low prices from export to S.E. Asia.

In this talk the long term viability of the fishery will be discussed. Strategies to mitigate the effects of overfishing including better management of the quota system, roe enhancement and reseedling will also be discussed.

**ENHANCEMENT OF YIELD AND QUALITY OF *EVECHINUS CHLOROTICUS* ROE THROUGH CONTROLLED DIET.** M. F. Barker<sup>1</sup>, P. Bremer<sup>2</sup>, P. Silcock<sup>2</sup>, C. Delahunty<sup>2</sup>, and M. Sewell<sup>3</sup>. <sup>1</sup>Department of Marine Science, <sup>2</sup>Department of Food Science University of Otago, Box 56, Dunedin, New Zealand, <sup>3</sup>School of Biological Sciences, University of Auckland, Auckland, New Zealand.

Export of New Zealand sea urchins; roe to Japan and other south-east Asian countries has been unsuccessful as the fishery does not consistently produce roe with marketable qualities. Previous research in our laboratory has shown that by holding animals in the laboratory or in sea cages, and feeding a gelatin bound or Wenger diet for ~8 to 12 weeks the yield of Kina roe can be significantly enhanced although the colour of such roe was often highly variable. The influence of diet on the colour or flavour of the roe was not quantitatively assessed. The present research was initiated to: 1) develop local diet formulation in commercial quantities, and 2) gain a better understanding of the relationship between the sensory and biochemical characteristics of urchin roe and the animals' diet.

Gonad data quantified to date include: gonad index 23–24%; colour (58.6–66.9° hue angle, 17.3–34.8 chroma); glucose concentration (0.1–0.8% dry weight basis (dwb)); glycogen concentration (8.5–16.6% dwb); and free amino nitrogen concentration (1.4–2.5% dwb). Gonad indices are significantly higher in urchins fed artificial compared to algal diets but not higher than animals collected from the wild. Free amino acids are believed to contribute significantly to the taste of sea urchin roe. We have determined that glycine is present in the highest concentration followed by

alanine and valine, though the relative amount of individual free amino acids varied between roes from different animals. We are also carrying out tests to determine the impact of handling and storage regimes on the shelf-life of *E. chloroticus* roe.

**PARTICULATE MATTER PRODUCTION ASSOCIATED WITH DEEP-WATER SUSPENDED PACIFIC OYSTER (*CRASSOSTREA GIGAS*) FARMS IN BRITISH COLUMBIA.**

P. A. Barnes, W. Friesen and S. Switzer, Centre for Shellfish Research, Malaspina University-College, 900 5th St., Nanaimo, B.C. V9R5S5, Canada.

The economic importance of bivalve culture in coastal areas of the northeastern Pacific is not reflected in the amount of information available on the environmental interactions of culture facilities. This study was designed to determine the fate of the particulate material produced by deep-water, suspended Pacific oyster farms in British Columbia. Data was collected quarterly for one year at two sites of contrasting current regimes. Seven stations, including two reference stations, were sampled at each site along a transect running through the centre of the oyster farm. Sediment trap data yielded deposition rates, including rates for TOC/TIC and TN. Chemical and biological parameters were measured on sediments at all sites. Water column data collected include plankton community composition, particle size and abundance, and a suite of environmental variables including salinity, temperature, dissolved oxygen, pH, turbidity/total suspended solids and chlorophyll. The results of preliminary analyses investigating relationships between depositional data, water column variables and benthic data for the seven stations at each of the two sites are discussed, in addition to seasonal trends.

**INVERTEBRATE BIODIVERSITY ASSOCIATED WITH DEEP-WATER PACIFIC OYSTER (*CRASSOSTREA GIGAS*) FARMS IN BRITISH COLUMBIA, CANADA.** P. A. Barnes<sup>1</sup>,

S. Switzer<sup>1</sup> and B. Burd<sup>2</sup>. <sup>1</sup>Centre for Shellfish Research, Malaspina University-College, 900 5th St., Nanaimo, B.C. V9R5S5, Canada, <sup>2</sup>Eco-Stat Research Ltd.

Diverse invertebrate communities ("fouling" communities) are associated with B.C.'s deep-water, suspended shellfish culture systems. The three-dimensional structure of these systems increases habitat complexity and provides hard substrate for larval settlement. These diverse communities require investigation to determine their role in the environmental interactions of shellfish aquaculture, to document invasive species and to assist in documenting B.C.'s coastal marine biodiversity. This study monitored fouling

communities associated with deep-water suspended oyster rafts at two sites of contrasting current regimes. Biodiversity data were collected in the summer, winter and spring; organisms were identified to species, or the lowest taxon possible using the current taxonomic literature. Community composition data are presented, along with results of preliminary data analysis investigating changes in community composition with depth, site and season.

#### HOW EFFICIENT IS THE BLUE MUSSEL (*MYTILUS EDULIS* L.) AT FILTERING EXCESS PARTICULATE MATERIAL AT AN INTEGRATED AQUACULTURE SITE?

Aaron T. Bennett<sup>1</sup>, Bruce A. Macdonald<sup>1</sup> and Fred H. Page<sup>2</sup>.

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In 2001, a collaborative project investigated the viability of integrated aquaculture (finfish/shellfish/seaweed) in the Bay of Fundy. The project will help to quantify the economic and environmental benefits and to serve as a model for developing integrated, sustainable aquaculture. The present study has two main objectives: 1) characterizing the suspended particle field within and surrounding an integrated aquaculture site using various methods to capture the entire size range of suspended particulates and 2) determining the uptake and absorption of suspended particles on the integrated aquaculture site by the blue mussel, *Mytilus edulis*. We are using videography to capture the size distribution of undisturbed particles from a minimum of 100 µm to the upper range of particle size limit. Particles smaller than the range of the video resolution are measured using a Coulter Multisizer which results in a size distribution pattern for individual particles measured. In order to obtain a more complete picture, a biodeposition rate is calculated from the feces and pseudofeces deposited by the mussels during the feeding trials. A net rate is then calculated for the amount of particulates completely removed from the system. We believe this research will support the assumption that mussels play a vital role in the environmental sustainability of integrated aquaculture in the Bay of Fundy.

#### OPTIMIZATION OF GROW-OUT CONDITIONS FOR THE LARGE SCALE CULTURE OF QUEEN CONCH, *STROMBUS GIGAS*. Anne Boettcher<sup>1</sup>, Julie Davis<sup>2</sup> and Catherine Dyer<sup>2</sup>.

<sup>1</sup>University of South Alabama, Biology Department, USCB 124, Mobile, AL 36688, <sup>2</sup>Caicos Conch Farm, Trade Wind Industries.

Queen conch has long been a staple within the Caribbean, and wild product is exported to an ever increasing global market. However, rising fishing pressure has resulted in the depletion of wild stocks which has increased interest in both research and commercial based aquaculture programs. The Caicos Conch Farm, a facility involved in the commercial-scale culture of queen conch, has

well established hatchery and juvenile rearing techniques and is in the process of optimizing onshore and offshore systems for the grow-out of conch. Recent studies focused on evaluation of pelleted test diets and determinations of feed conversion ratios. Increases in conch growth are seen with the inclusion of natural algal components to feed, and pellet formulations that include an alga base or algal mimetics are being developed. Feed conversion ratios similar to those seen for other aquaculture species are achieved with the current pelleted diet. In conjunction with an evaluation of onshore pond systems and offshore pen design, stocking densities and transfer protocols for conch in these systems are being evaluated. The technology developed through these studies will be implemented in a new grow-out facility on Grand Turk.

#### APPLICATION OF PHOTOPERIOD MANIPULATION AND NEW EXTRUDED DIETS IN SEA URCHIN AQUACULTURE. S. Anne Boettger<sup>1</sup>, Charles W. Walker<sup>1</sup>, Michael G. Devin<sup>2</sup>, Stephen A. Watts<sup>3</sup>, Mickie L. Powell<sup>3</sup> and Addison L. Lawrence<sup>4</sup>.

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For the past two decades, sea urchin fisheries have become important commercial resources with US exports to Japan exceeding \$150 million in 1995. However, sea urchin populations along the coasts of the US have been overexploited. In the largest US urchin fishery (State of Maine), landings and numbers of licensed urchin fishermen have declined between 1993 and 2001. To develop new aquaculture techniques and more effective diets we have used information on cellular changes occurring during gametogenesis. Individuals were exposed to ambient and July photoperiod, fed an extruded diet (Wenger) and were evaluated for gonad index, stereology of different gonadal cells, nutritive phagocyte and oocyte sizes and taste. Gonad indices (%) increased significantly in both treatments over five months. Amounts of nutritive phagocytes (%) increased significantly only under invariant photoperiod while sizes (µm) of nutritive phagocytes increased in both photoperiod treatments but were significantly greater at invariant photoperiod. Amounts of gonial cells (%) increased significantly under ambient photoperiod. Oocyte feret diameters (µm) increased in both treatments but were largest at ambient photoperiod. Gonad (roe) taste was not appealing to taste testers. Therefore, seven diets of varying protein concentrations and the Wenger diet were fed to individuals maintained at invariant photoperiod for five months. This resulted in increased gonad indices in all diet treatments, but enhanced marketability of individuals for only three diets all of low/medium protein and high carbohydrate composition. Our results indicate that photoperiod manipulation and new diets can yield marketable sea urchin roe.

# AN EXAMINATION OF THE BIOTIC AND ABIOTIC FACTORS INFLUENCING THE PARASITIC RELATIONSHIP OF A RHIZOCEPHALAN BARNACLE AND ITS HOST.

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In the Gulf of Mexico and adjacent waters, the rhizocephalan barnacle *Loxothylacus texanus* parasitizes members of the genus *Callinectes*. However, factors affecting the distribution and prevalence of this parasite are not well understood. A series of studies have been conducted to determine what biological and physical factors influence this host-parasite relationship. *L. texanus* larvae are non-feeding and have approximately three days from metamorphosis to the cyprid stage for identification of a host, before their nutrient stores are depleted. During this time cypris larvae appear to be particularly sensitive to abiotic variations, including salinity fluctuations. The actual identification of a potential host appears to be mediated by chemical cues associated with the host, similar to those used by free-living barnacles. The female cypris larvae of *L. texanus* settle in response to carbohydrate or glycoprotein cues in the epicuticle layer of crab exoskeletons. Laboratory studies indicate, however, that successful settlement does not guarantee infection of the host. Results suggest that infection rates may be limited by factors such as host size, carapace content or immune response.

# MYTELLA CHARRUANA ALONG THE ATLANTIC COAST OF FLORIDA: A SUCCESSFUL INVASION? Michelle Boudreaux, Nancy Gillis and Dr. Linda Walters. University of Central Florida.

Biological invasions are recognized as one of the most serious problems confronting the integrity of native species and ecosystems around the world. Unfortunately, invaders often go unnoticed until they have spread extensively, making eradication difficult and very costly. Early detection and rapid response to invasions are vital to prevent potential evolutionary and ecological changes that damage both our ecosystems and our economy. *Mytella charruana*, a tropical mussel native to Mexico and South America, first appeared in the intake pipes of a Jacksonville, Florida power plant in 1986. Fortunately, they never became established as the founder population was extirpated the winter of 1987, presumably due to cold temperatures. No new sightings of this species were recorded until 2004. Then, a population of *M. charruana* was discovered in Mosquito Lagoon, Florida (170 km south of Jacksonville) in August 2004. Since then the area has been surveyed monthly for mussel occurrences. Specimens were found August 2004–February 2005 on manmade debris, driftwood and living oysters. No individuals were found again until August 2005. If ecological conditions are optimal for continued survival and establishment of *M. charruana*, this species has the potential to reproduce and out-

compete native mussels and declining oyster populations of the area. Our goal is to better understand this invasive before this happens. Rapid actions are needed to prevent *M. charruana* from having the economic and ecological impacts of the zebra mussel *Dreissena polymorpha* and green mussel *Perna viridis*.

# SPATIAL COMPETITION BETWEEN OYSTERS AND BARNACLES IN A FLORIDA ESTUARY. Michelle Boudreaux and Dr. Linda Walters. University of Central Florida.

Populations of *Crassostrea virginica* within Mosquito Lagoon, Florida have recently undergone significant die-offs. Before effective restoration protocols can be established, important questions about the ecology of these oysters must be answered. My research focused on interactions between oysters and spatial competitors that may affect the settlement, growth, and survival of *C. virginica*.

I collected species inventory data for one year using lift nets to determine the sessile species present. Barnacles (*Balanus amphitrite* and *B. cernuus*) were the most abundant competitors. To determine if *Balanus* spp. were always in competition with oysters, shells from historic shell middens were examined. Results show that there has been a five fold increase in barnacle abundance per oyster shell and appearance of an invasive barnacle, *B. amphitrite*, since midden shells. Thus, spatial competition between barnacles and oysters has increased in Mosquito Lagoon.

To determine how *Balanus* spp. affected settlement, growth, and survivorship of *C. virginica*, experiments were conducted in which densities of *B.s amphitrite* and *B. cernuus* were manipulated on disarticulated oyster shells. Density treatments included: no barnacles (control), low, medium, and high coverage of barnacles. Laboratory settlement trials with oyster larvae were run with all shell treatments. Settlement was counted by microscopy. Treatments with oyster spat were deployed in the field to follow oyster growth and survivorship over 4 weeks. Settlement, growth, and survivorship of oysters were all affected by the presence of barnacles even in low densities. The specific *Balanus* spp. did not have an effect on oyster settlement, growth, or survivorship.

# JUVENILE RECRUITMENT DYNAMICS OF THE PINTO ABALONE (*HALIOTIS KAMTSCHATKANA*) IN THE SAN JUAN ISLAND ARCHIPELAGO, WASHINGTON. Joshua V. Bouma<sup>1</sup>, C. Jackels<sup>1</sup>, K. M. Straus<sup>1</sup>, B. Vadopalas<sup>1</sup>, C. S. Friedman<sup>1</sup> and D. P. Rothaus<sup>2</sup>. <sup>1</sup>University of Washington, School of Aquatic and Fishery Science, Box 355020, Seattle, WA 98195. <sup>2</sup>Washington Department of Fish and Wildlife.

Research has shown that adult pinto (northern) abalone (*Haliotis kamtschatkana*) are in serious decline, prompting designation as a Threatened Species (Canada) and a Species of Concern (Washington State; U.S.). These declines may be due to recruitment failure; thus more information is needed about the life history of this species, especially larval and juvenile stages. We deployed



60 abalone recruitment modules (ARMs) previously shown to successfully attract juvenile abalone less than 50 mm shell length in the wild, at two depths and three sites in the San Juan Island archipelago (SJI) with historically significant abalone populations. All ARMs were surveyed *in situ* for juvenile abundance three times over the course of 15 months. No juvenile abalone were observed, although the ARMs were successful in attracting adult abalone and other juvenile invertebrate and fish species. The lack of juvenile abalone in the ARMs may be due to Allee effects or, in the northern SJI, to elevated temperatures and lowered salinities observed from the Fraser River summer plume. To test the latter hypothesis, a  $3 \times 3$  full factorial experiment was implemented to examine the influence of environmentally relevant temperature (11°C, 16°C, and 21°C) and salinity (14 ppt, 23 ppt and 32 ppt) combinations on post-larval (3–7 mm) pinto abalone survival. By day three of the experiment 100% mortality was observed in all 14 ppt treatment groups regardless of temperature. Mortalities in each of the remaining temperature/salinity combinations over the 14 day study were not significantly different than controls, suggesting intolerance to low salinity in this species.

**FOULING IN SCALLOP CULTIVATION: SOME ISLE OF MAN EXPERIENCES.** Andrew R. Brand and Katherine A. Ross. Port Erin Marine Laboratory, University of Liverpool, Port Erin, Isle of Man, IM96JA, UK.

Fouling of scallop shells and cultivation nets by living organisms can reduce scallop growth and is costly to remove. We examined the effects of fouling on physiochemical and food conditions inside pearl nets used for suspended culture of the great scallop (*Pecten maximus*) in the Irish Sea, off the Isle of Man, and also investigated biological control of fouling using a range of invertebrates. Fouling of nets reduced water movement but, contrary to common assumptions, did not reduce the quantity or the quality of food particles available for scallops. Other negative effects of fouling, such as accumulation of inorganic matter, nitrate and ammonia, were also absent. We concluded that the negative effects of fouling found in some previous studies may have been caused by foulers parasitizing or mechanically interfering with scallops, rather than creating an unfavourable environment. The biological control trials found that sea urchins (*Echinus esculentus* and *Psammechinus miliaris*) and hermit crabs (*Pagurus* sp.) removed fouling from nets more efficiently than other invertebrates. In our short-term experiments only *P. miliaris* was associated with increased scallop shell growth, but no biological control organism reduced scallop growth or survival. These results suggest that biological control could be an efficient and environmentally sound method of dealing with the problem of fouling in scallop cultivation.

**CRASSOSTREA ARIAKENSIS AND C. VIRGINICA RESPONSES TO ICHTHYOTOXIC KARLODINIUM VENEFICUM.** E. F. Brownlee<sup>1</sup>, A. R. Place<sup>2</sup>, H. Nonogaki<sup>2</sup>, J. E. Adolf<sup>2</sup>, S. G. Selner<sup>3</sup> and K. G. Selner<sup>4</sup>. <sup>1</sup>Calvert High School, <sup>2</sup>Center for Marine Biotechnology, University of Maryland, <sup>3</sup>Morgan State Environmental Research Center, <sup>4</sup>Chesapeake Research Consortium.

The Eastern oyster, *Crassostrea virginica*, and the Asian oyster, *C. ariakensis*, are native and potentially introduced oysters, respectively, in the Chesapeake Bay and as such, will be exposed to the natural phytoplankton assemblages including harmful species throughout their life cycles. Recent work suggests that at least one of these prey items, the ichthyotoxic dinoflagellate *Karlodinium veneficum*, occurs frequently throughout the growth period for the oysters, thereby insuring frequent exposures for all life stages of the bivalve. *K. veneficum* produces linear polyketide toxins (karlotoxins), which elicit toxicity through sterol-dependent, non-specific pores in biological membranes. Spat and juvenile oysters of each species were exposed to a moderately toxic level [ $18.5 \pm 6.2$  ng ml<sup>-1</sup>] of the dinoflagellate at environmentally-relevant cell densities and growth contrasted with rates observed on other phytoplankton species, including the spring bloom former *Prorocentrum minimum* and a phytoplankton mixture routinely used in oyster hatcheries. Growth for spat and juveniles of both oysters was significantly reduced when feeding on *K. veneficum*, relative to the other prey species. Experiments are continuing to identify the importance of food quality versus toxin content in oyster responses. These initial results suggest that the cosmopolitan *K. veneficum* inhibits growth and would potentially curtail oyster production within the tidal Bay and its tributaries throughout the oysters' growth periods and considering its global distribution, potential impact throughout temperate areas should be assessed.

**FILTRATION CAPACITY AS A WAY TO SCALE RESTORATION OBJECTIVES: AN EXAMPLE FROM CHESAPEAKE BAY.** Robert D. Brumbaugh<sup>1</sup> and A. Thomas Leggett Jr.<sup>2</sup> <sup>1</sup>The Nature Conservancy Global Marine Initiative, URI Narragansett Bay Campus, South Ferry Rd., Narragansett, RI 02883, <sup>2</sup>Chesapeake Bay Foundation.

The decline of eastern oyster populations in Chesapeake Bay and elsewhere in the species' range is well documented, and efforts are underway to develop and test restoration approaches. In addition to restoring lost fisheries productivity, ecosystem services are increasingly cited as a reason for restoration. Policy makers in the Chesapeake Bay region have adopted a bay-wide goal for oyster restoration (10% of historic abundance by 2010), but little guidance exists for forecasting exactly how much restoration effort will be necessary to achieve this goal. We used the Lynnhaven River, a small tidal tributary in the southern Chesapeake Bay, as a model



for both evaluating the efficacy of restoration approaches and developing scaling arguments for the larger Bay-wide restoration effort. Oyster restoration in the Lynnhaven began in 1998 and has entailed both reef habitat enhancement (~5.5 acres) and stock enhancement to increase recruitment rates. Oyster biomass, estimated from annual abundance surveys, was used to calculate a “filtration capacity” of restored reefs. We estimate that oysters on the restored reef area are capable of filtering a volume equivalent to the river’s volume every 63.5 days. This approach can be used to set restoration goals at scales ranging from tributaries to entire estuaries.

**IMPACTS OF THE COLONIAL ASCIDIAN (*DIDEMNUM SP. A*) ON MUSSELS, OYSTERS AND SCALLOPS.** **Stephan G. Bullard<sup>1</sup>, Robert B. Whitlatch<sup>2</sup>, Richard W. Osman<sup>3</sup> and Sandra E. Shumway<sup>2</sup>.** <sup>1</sup>University of Hartford, Hillyer College, 200 Bloomfield Ave., West Hartford, CT 06117. <sup>2</sup>University of Connecticut. <sup>3</sup>Smithsonian Environmental Research Center.

The carpet tunicate (*Didemnum sp. A*) is a colonial ascidian of unknown origin with rapidly expanding populations on the east and west coasts of North America. The carpet tunicate can grow on most natural and man-made hard substrata habitats (rocks, cobbles, docks, pilings, boat hulls). Large colonies form nearly solid mats that may smother benthic organisms. The carpet tunicate may be of particular concern for shellfish, and thus the aquaculture industry, as colonies can overgrow individual bivalves or completely cover material used to culture them. To determine the effect of the carpet tunicate on cultured juvenile shellfish, we deployed mussels (*Mytilus edulis*), oysters (*Crassostrea virginica*), and scallops (*Argopecten irradians*) in bags at 3.5 m depth with and without fragments of *Didemnum sp. A*. We assessed the survivorship, growth and condition index of shellfish after approximately one and two months of deployment. After one month, many shellfish in the treated bags had the ascidian growing on their shells; some were 100% covered. Preliminary data on survivorship, growth and condition index will be presented.

**A MOLECULAR COMPARISON OF OYSTER HERPES VIRUSES FROM ASIA, EUROPE, AND NORTH AMERICA.** **Colleen A. Burge<sup>1</sup>, Carolyn S. Friedman<sup>1</sup>, Kimberly S. Reece<sup>2</sup>, Jessica A. Moss<sup>2</sup> and Tristan Renault<sup>3</sup>.** <sup>1</sup>University of Washington, School of Aquatic and Fishery Sciences, Seattle, WA 98195. <sup>2</sup>Virginia Institute of Marine Science, <sup>3</sup>IFREMER.

Oyster herpes viruses (OsHV) have been identified in multiple bivalve species globally. Some of these herpes viruses have been identified and described using molecular tools including Polymerase Chain Reaction (PCR), *in situ* hybridization (ISH), and nucleic acid sequencing. To date, PCR and sequence analysis have identified possible variants. One variant (OsHV-1) collected in France

was recently purified, sequenced, and morphologically characterized as a member of the family *Herpesviridae*. We have begun using PCR primer sets developed to amplify distinct regions of the OsHV-1 genome to identify possible sequence divergences in OsHV in oysters collected from two sites in China (*Crassostrea ariakensis*), one site in Japan (*C. gigas*), and one site in the United States (*C. gigas*) as compared to OsHV-1. Preliminary PCR results indicate that the OsHV identified in China and Japan is similar, but differs from OsHV-1, while samples collected in the United States are more comparable to OsHV-1. We plan to expand this analysis to include samples from sites in China and Korea where OsHV has been identified. We also plan to test additional primer sets and to sequence additional regions to better describe OsHV variants.

**EASTERN OYSTER SETTLEMENT AND EARLY SURVIVAL ON ALTERNATIVE SUBSTRATES ALONG INTERTIDAL MARSH, RIP RAP, AND MANMADE OYSTER REEF.** **Russell Burke<sup>1</sup>, Romuald Lipcius<sup>1</sup>, Mark Luckenbach<sup>1</sup>, P. G. Ross<sup>1</sup>, Justine Woodward<sup>1</sup> and David Schulte<sup>2</sup>.** <sup>1</sup>Virginia Institute Marine Science: College of William & Mary, 1208 Greates Rd., Gloucester Point, VA 23062, <sup>2</sup>Army Corps of Engineers—Norfolk, VA District.

Restoration efforts with native Eastern oyster, *Crassostrea virginica*, in Chesapeake Bay have been extensive and have been impeded by substrate and recruitment limitations along with many other detrimental factors. In Lynnhaven Bay, a southern subestuary of Chesapeake Bay, the Army Corps of Engineers has partnered with the Virginia Institute of Marine Science, National Oceanic and Atmospheric Administration, and Chesapeake Bay Foundation to implement a comprehensive oyster reef restoration strategy. Surveys within the Lynnhaven Bay system indicate that artificial oyster shell reefs created in the early 1990s are producing poor to marginal oyster densities relative to densities on nearby granite and concrete riprap, and on oyster clusters along marsh-fringed shores. In this field experiment, twelve treatments simulating inter-tidal oyster habitat were placed at three sites within a tidal creek: adjacent to marsh, rip rap and a manmade oyster reef. Treatments consisted of caged and uncaged trays (0.5 m length × 0.5 m width × 0.25 m depth) of large granite, small granite, large limestone marl, small limestone marl, very small concrete/granite, and oyster shells. Granite of both sizes had the highest initial oyster recruitment across all sites. Overall, replicates showed a distinct recruitment/early survival pattern between sites: marsh > rip rap > artificial oyster reef. We therefore propose that granite may be a favorable oyster reef construction material, since it appears to enhance oyster settlement and early post-settlement survival. Additional biological benefits may accrue from granite reefs as community structure develops on the reefs.

**DIVERSITY AND DISTRIBUTION OF UNIONID BIVALVES IN TEXAS.** Lyubov E. Burlakova<sup>1</sup>, Alexander Y. Karatayev<sup>1</sup>, Robert G. Howells<sup>2</sup> and Daniel L. Bennett<sup>1</sup>. <sup>1</sup>Department of Biology, Stephen F. Austin State University, Box 13003—SFA Station, Nacogdoches, TX 75962-3003, <sup>2</sup>Texas Parks and Wildlife Department, Ingram, TX.

The family *Unionidae* is one of the most rapidly vanishing faunal groups in North America, and habitat destruction is considered to be the most important cause for the decline. To evaluate the current status of unionid diversity in Texas we re-examined species composition and abundance of unionid in 30 waterbodies previously surveyed by the Texas Parks and Wildlife Department. The highest diversity was found in Sabine River (18 species), B. A. Steinhagen Reservoir (16), Neches River below Town Bluff Dam (14), Village Creek (14), and Sanders Creek (14). The lower San Marcos River and Guadalupe River (Central Texas) were found to support rare, endemic *Quadrula aurea*. Living rare endemic *Lampsilis bracteata* were found in Live Oak Creek, the Guadalupe River, and the San Saba River (Central Texas). Rare species of *Fusconaia* (*F. askewi* and endemic to Texas *F. lananensis*) are still present in Attoyac Bayou and Sandy Creek, and in Village Creek in Big Thicket National Preserve (East Texas). In addition, this latter creek continues to support rare *Pleurobema riddelli*. Several large reservoirs surveyed had drought- and drawdown-related low waters in recent years that presumably caused reductions in mussel abundance and diversity.

**CHANNELED-TYPE APPLESNAILS: CURRENT DISTRIBUTION, DENSITIES AND POTENTIAL THREAT TO NATURAL ECOSYSTEMS AND AGRICULTURE.** Lyubov E. Burlakova, Alexander Y. Karatayev, David N. Hollas and Leah D. Cartwright. Department of Biology, Stephen F. Austin State University, Box 13003—SFA Station, Nacogdoches, TX 75962-3003.

The aquatic invasive gastropod *Pomacea canaliculata* (channeled applesnail), originally from South America, has become a major pest of rice crops throughout the Indo-Pacific Region. These large herbivorous snails can reach maturity in about two months during the summer, reproduce several times a month, and aestivate for several months, burying into the soil if the habitat dries out. By mid 2005, living populations of channeled-type applesnails were reported from Florida, Texas, California, Alabama, Arizona, and Georgia. According to the results of genetic analyses, specimens from Texas and Florida belong to the *P. canaliculata*-group. The first reproductive applesnail population in Texas was reported in 1989, and by the summer of 2005 snails were found in six south-eastern counties (Harris, Chambers, Brazoria, Galveston, Fort Bend, and Waller). Reproduction of channeled-type applesnails in Texas continues for at least 8 months, from March to the beginning of November. Densities of snails in Texas vary depending on waterbody type and possibly presence of predators. The rice grow-

ing technology used in Texas combined with current low densities of snails are the likely reasons why agricultural damage due to applesnails has not yet been detected.

**OYSTER SEX WARS: EVIDENCE FOR A 'GAMETE SINK' IF *CRASSOSTREA VIRGINICA* AND *CRASSOSTREA ARIAKENSIS* SPAWN SYNCHRONOUSLY.** David Bushek, Andrea Kornbluh, Greg Debrosse, Haiyan Wang, Ximing Guo and John Quinlan. Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Sciences, Rutgers, The State University of New Jersey, 6959 Miller Avenue, Port Norris, NJ 08349 USA.

Available data indicate spawning seasons for the Asian oyster *Crassostrea ariakensis* and the eastern oyster *C. virginica* overlap. Hybrids can form, but the larvae are not viable. If *C. ariakensis* are introduced into Chesapeake Bay and synchronous spawning occurs with *C. virginica*, hybridization could reduce the production of viable larvae (= gamete sink). We examined the effects of gamete age, sperm concentration, and ratios of heterospecific gametes on fertilization rate and hybridization of the two species. Hybrid fertilization rates were consistently lower than pure crosses. Fertilization rate decayed with gamete age, but occurred in gametes up to eight hours old. Fertilization rate also decayed with decreasing sperm density in both pure and hybrid crosses. Finally, fertilization rate declined by as much as 60% when sperm were (1) given a choice of eggs from each species to fertilize or (2) required to compete to fertilize eggs from a single species. Hence, a gamete sink will likely occur if these two species spawn synchronously. The magnitude of the gamete sink will depend in part on proximity of the two species, on gamete concentrations in the water column, and on the proportion of hybrids that form. Current efforts are enumerating the proportion of hybridization that occurred during these experiments. Molecular genetic methods to amplify ITS regions of the rRNA gene have been validated and yield two bands in pure crosses and four bands in hybrid crosses. Individual larvae are being typed to determine the proportion of hybrids formed under the various gamete mixtures.

**ESTIMATION OF RECREATIONAL DUNGENESS CRAB HARVEST IN PUGET SOUND, WASHINGTON USING A TELEPHONE SURVEY OF HARVESTERS.** Jennifer Cahalan. Washington Department of Fish and Wildlife, Point Whitney Shellfish Laboratory, 1000 Point Whitney Road, Brinnow, WA 98320.

The recreational Dungeness crab harvest in Puget Sound exceeds 1.5 million pounds of crab annually. In order to meet fisheries management decision-making needs, estimates of recreational Dungeness crab harvest need to be precise and accurate, provided for relatively small geographic and temporal scales, and

available during the recreational season. The recreational Dungeness crab fishery is characterized by relatively low effort dispersed over a large geographic area. As a result field-based methods of estimating recreational catch were inefficient and expensive. Alternate methods of estimating catch were developed and implemented in 2002.

To fish for crab in Washington State, crab harvesters are required to obtain a catch record card (CRC) from the WDFW. A telephone survey of CRC-holders is used to collect the data used in catch estimation. Catch estimates are generated approximately three to four weeks after the survey begins for each of Puget Sound's 11 catch areas. A minimum of two surveys are conducted each season. This method provides timely in-season estimates of harvest at relatively low cost. In this presentation, the details of the methods will be discussed, including treatment of missing data, precision of estimates, cost, and potential improvements.

**INBREEDING EFFECTS ON GROWTH AND SURVIVAL IN A NATURALIZED POPULATION OF THE PACIFIC OYSTER (*CRASSOSTREA GIGAS*) REVEALED USING MARKER BASED RELATEDNESS ESTIMATES.** Mark D. Camara<sup>1</sup>, Sanford Evans<sup>2</sup>, and Chris Langdon<sup>2</sup>. <sup>1</sup>USDA—Agricultural Research Service, 2030 SE Marine Science Dr., Newport, OR 97365, <sup>2</sup>Molluscan Broodstock Program, Newport, Oregon USA.

Inbreeding is an important factor influencing mating systems, dispersal mechanisms, and variation in life history traits within and among populations and thus has profound implications in evolutionary biology, selective breeding, conservation biology, and even medicine. In theory, high fecundity species, such as elm trees and oysters, are expected to have higher genetic load and consequently more severe inbreeding depression than low fecundity species, and previous studies have confirmed that self-fertilization, brother/sister matings, and cousin/cousin matings in *C. gigas* produce progeny with lower growth and survival than non-consanguineous pairings of parents. It is unclear, however, whether these effects can be extrapolated to the lower levels of consanguinity expected in natural populations with random mating. We studied inbreeding in a naturalized population of Pacific oysters using three molecular marker-based estimates of parental relatedness calculated from multi-locus microsatellite genotypes. Using analysis of covariance approaches, we found significant relationships between estimators of parental genetic similarity and components of fitness. As expected, more highly related parents produced progeny with lower survival (i.e. inbreeding depression). Surprisingly, however, the surviving progeny of genetically similar parents had higher growth (outbreeding depression or heterosis) than less related parental pairs. We discuss potential mechanism underlying these patterns and their implications for aquaculture and selective breeding.

**HAEMOLYMPH PROTEOMIC APPROACH TO ANALYSE DIFFERENCES IN SUSCEPTIBILITY TO BONAMIOSIS BETWEEN OYSTER STOCKS.** Asunción Cao, José Fuentes and Antonio Villalba. Centro de Investigacións Mariñas, Xunta de Galicia, P.O. Box 13, 36620 Vilanova de Arousa, Spain.

The parasitic protozoan, *Bonamia ostreae*, causes oyster, *Ostrea edulis*, mass mortalities throughout the European Atlantic coast. The parasite is phagocytosed by oyster haemocytes but is not killed. The parasite divides in haemocyte cytoplasm causing the rupture of the host cell. Differences in susceptibility to bonamiosis between *O. edulis* stocks have been verified. Furthermore, the oyster *Crassostrea gigas* is resistant to bonamiosis. Separation of the proteins of oyster haemolymph by two-dimensional electrophoresis was tuned up resulting in multiple proteic spots on polyacrilamide gels. This proteomic approach is being applied to analyse differences in susceptibility to bonamiosis between *O. edulis* stocks and between *O. edulis* and *C. gigas*. The *O. edulis* stocks used in the comparisons are: Rossmore (strain selected for resistance against bonamiosis), oysters from Tralee Bay (Ireland) where the parasite had not previously been detected, and oysters from Ría de Ortigueira (Galician, NW Spain) where *B. ostreae* is present since early 1980s. Comparison between infected and uninfected oysters is also included. Application of specific commercial software makes easy qualitative and quantitative comparison between gels corresponding to haemolymph of each oyster group. Early results have shown spots exclusive of each group and other spots that are common to every oyster group. Identification of the proteins in the spots could contribute to explain differences in susceptibility and even provide the key for resistance to bonamiosis.

**POTENTIAL IMPACT OF *BONAMIA* SP. ON *CRASSOSTREA ARIAKENSIS* IN CHESAPEAKE BAY AND NORTH CAROLINA.** Ryan B. Carnegie<sup>1</sup>, Nancy A. Stokes<sup>1</sup>, Corinne Audemard<sup>1</sup>, Eugene M. Burreson<sup>1</sup>, Melanie J. Bishop<sup>2</sup>, Charles H. Peterson<sup>3</sup>, Ami E. Wilbur<sup>3</sup>, Troy D. Alphin<sup>4</sup> and Martin H. Posey<sup>4</sup>. <sup>1</sup>Virginia Institute of Marine Science, <sup>2</sup>University of North Carolina Institute of Marine Sciences, <sup>3</sup>UNC Wilmington Center for Marine Sciences, <sup>4</sup>UNC Wilmington Dept. of Biology and Marine Biology.

A *Bonamia* sp. emerged in 2003 as the cause of mortality (>85%) among experimental seed *C. ariakensis* in Bogue Sound, North Carolina. With introduction of this oyster to Chesapeake Bay proposed, this epizootic gave urgency to investigations into the nature of bonamiasis in the mid-Atlantic: its annual timing and range, environmental limitations, and the identity and distribution of parasite reservoirs. Field studies included *Bonamia* sp. monitoring in *C. ariakensis* deployments along a salinity gradient from Bogue Sound, and at distant coastal sites; in serial deployments of seed *C. ariakensis* to upwellers on Bogue Sound; and among *Ostrea equestris* from Bogue Sound and Wilmington, NC. Labo-

ratory trials have explored effects of reduced salinity on existing *Bonamia* sp. infections. Epizootics recurred in 2004–2005, generating infections for laboratory use and allowing an initial portrait of *Bonamia* sp. to be developed. This parasite causes acute disease and mortality in smaller/younger *C. ariakensis* (<40 mm) in higher salinity coastal waters during warmer months. Infections may regress in winter. It is known to occur only at Bogue Sound and Wilmington, >100 km to the southwest. It occurs in *O. equestris* in both places, but this oyster may not be a functional reservoir for *Bonamia* sp., or its only other host. In the laboratory, infections were purged at 20 and 10 psu, further suggesting that lower estuarine salinities (such as in Pamlico Sound, where *Bonamia* sp. has been notably absent from a mesohaline *C. ariakensis* nursery site, or Chesapeake Bay) may limit *Bonamia* sp. activity.

#### DEVELOPING STRATEGIES TO REDUCE THE IMPACT OF THE BORING SPONGE *CLIONA CELATA* ON CULTURED EASTERN OYSTERS *CRASSOSTREA VIRGINICA*.

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It has long been recognized that the boring sponge (*Cliona celata*) can cause significant economic losses to those involved in the harvesting of wild oyster populations. It is not clear, however, whether the presence of a boring sponge population may act as a source of infection to cultured oysters grown in floating bags or on oyster tables. Preliminary surveys conducted in one bay in northern New Brunswick, Canada, suggested that in some areas 35% of the off-bottom cultured oysters showed evidence of shell degradation associated with boring sponge infection. The development of strategies to mitigate the impact of this organism is hampered by the lack of basic information on growth rates, spawning activity, and modes of infection. We report on the preliminary results from an ongoing multi-year project to document the life-history characteristics of this biofouling species and evaluate the effectiveness of various treatment strategies.

#### PREVALENCE AND GEOGRAPHIC DISTRIBUTION OF A DUNGENESS CRAB (*CANCER MAGISTER*) MICROSPORIDIAN PARASITE.

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The prevalence and geographic distribution of *Nadelspora canceri* a microsporidian parasite of the Dungeness crab *Cancer magister*, was studied and prevalences were analyzed by sex and size of host and date of collection. *N. canceri* was widely distributed along the United States Pacific Coast in estuaries from Bodega Bay, California to southern Washington. Prevalence of the parasite

was high in crabs from most estuaries sampled between 1991 and 1994, ranging from a low of 0.4% (Gray's Harbor, Washington) to 41.2% (Tillamook Bay, Oregon) with an overall mean of 14%. Crab examined from Grays Harbor between 2002 and 2004 indicate the prevalence rate had increased from 0.4% to greater than 14%. The parasite also occurred offshore of California and Oregon, but was not found in Puget Sound, Washington or in Glacier Bay, Alaska. The prevalence of infection in crabs captured in the open ocean was low (0.3%). In crabs collected nearshore at estuary mouths prevalence was intermediate between estuarine and open-ocean levels. The infection was most prevalent in two year old crabs (13 cm carapace width), and males had 2.5 times the rate of infection than did females.

The mortality of laboratory-held crabs naturally infected with *N. canceri* was compared to that of uninfected crabs and significantly higher mortality was observed for infected crabs. *N. canceri* infections were established in juvenile and adult crabs that were fed parasite spores in laboratory experiments indicating that transmission is direct and intermediate hosts or vectors are not required for transmission.

#### HABITAT ATTRIBUTES ASSOCIATED WITH BAY SCALLOPS IN AN AREA WITH ACTIVE RESTORATION.

Marnita M. Chintala<sup>1</sup>, Eric J. Weissberger<sup>1</sup>, David W. Grunden<sup>2</sup>, Elizabeth Hinchey<sup>3</sup>, William G. Nelson<sup>4</sup>, Russell Ahlgren<sup>1</sup> and Michael Charpentier<sup>4</sup>. <sup>1</sup>U.S. EPA, ORD/NHEERL, Atlantic Ecology Division, <sup>2</sup>Town of Oak Bluffs, MA, <sup>3</sup>Illinois-Indiana Sea Grant, Purdue University, <sup>4</sup>CSC Corporation, Narragansett, RI.

Habitat quality and quantity are important factors to maintain bay scallop (*Argopecten irradians*) populations, however, data linking habitat attributes to bay scallop populations are lacking. This information is essential to relate habitat alteration to the decline of bay scallop populations and to guide restoration efforts to reverse declines. Initial results of 30 dive surveys conducted in September 2005 in Lagoon Pond, Martha's Vineyard, MA, (538 acres) indicate a correlation between scallop abundance and total plant cover (macroalgae and eelgrass combined), depth, and sediment type. Scallop abundance was highest (15.42 scallops m<sup>-2</sup>) at a site where average total plant cover was 24%. Most scallops were found in shallower areas (depth 1–5 m) along the edge of Lagoon Pond, with deeper, unvegetated, sites devoid of scallops. Shell height (measured from umbo to opposite edge) ranged from 10 to 90 mm, with larger scallops present in the northern and eastern pond areas. Height distribution was primarily unimodal; however, a few locations had bimodal distributions. Scallop abundance will be related to restoration methods (e.g., seeding, spat bags) in the pond to assess how these activities have influenced the relationship between bay scallops and habitat.

**EFFECTS OF TRICLOSAN ON THE OYSTER PARASITE, *PERKINSUS MARINUS* AND ITS HOST, THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*: A COMPARISON AT DIFFERENT TEMPERATURES.** Fu-Lin E. Chu, Eric D. Lund, and Jennifer A. Littell. Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062, USA.

While causing minimal effect on oyster hemocyte viability, our previous study showed that the antibiotic, triclosan, not only inhibits growth and greatly reduces the viability of *in vitro* cultured *Perkinsus marinus* meronts, the primary disease transmission stage, but also inhibits the parasite's fatty acid synthetic ability. Due to the great importance of temperature on disease progression in the field, we tested the effects of triclosan on the viability of *P. marinus* meronts and oyster hemocytes at high and low temperatures. Parasite or oyster hemocytes were exposed to triclosan (0, 2, 5, or 10  $\mu$ M) for 24 hr and then their viabilities were assessed by MTS/PMS assay. Exposure of *P. marinus* meronts to 2, 5, or 10  $\mu$ M triclosan at 20, 26 and 28°C significantly reduced their viability (40–60%). At 13°C, *P. meronts* had a better triclosan tolerance, with viabilities of 67–73%. Oyster hemocytes treated with triclosan exhibited less than 10% mortalities at triclosan concentrations of  $\leq 10$   $\mu$ M at 13°C. Hemocytes viabilities reduced slightly (10–16%) at 5 and 10  $\mu$ M triclosan exposure. No significant reduction ( $\leq 0.05$ ) in viability relative to the controls occurred in hemocytes at triclosan concentrations from 2 to 10  $\mu$ M at 26°C. However, exposure of hemocytes to triclosan at 28°C, reduced viability to less than 63% at a concentration of 10  $\mu$ M. Triclosan exposure did not appear to affect reactive oxidative intermediate production in zymosan-stimulated and unstimulated hemocytes. This study is supported by a grant from the Oyster Disease Research Program (Grant # V710720), Sea Grant, NOAA.

**SC'S COMPREHENSIVE INTERTIDAL OYSTER ASSESSMENT USING HIGH RESOLUTION IMAGERY FOR MANAGEMENT AND RESTORATION.** L. D. Coen<sup>1</sup>, K. E. Schulte<sup>1</sup>, G. M. Yianopoulos<sup>2</sup>, R. E. Van Dolah<sup>1</sup>, W. D. Anderson<sup>2</sup>, M. A. Finkbeiner<sup>3</sup> and W. R. Stevenson<sup>3</sup>. <sup>1</sup>MRRI, SCDNR, 217 Fort Johnson Rd., Charleston, SC 29912, <sup>2</sup>OEM, SCDNR, <sup>3</sup>NOAA Coastal Services Center.

SCDNR is currently undertaking a state-wide assessment of its oyster resources as part of a multi-year, collaborative effort with NOAA's Coastal Services Center (CSC) and the U.S. Geological Survey. Using a previously developed approach for analyzing multispectral ¼ m digital imagery, we are processing the finalized imagery (digital orthophoto quarter quads) using Feature Analyst® and ERDAS's Imagine® to derive oyster reef location (presence-absence), areal extent and proportion of vertical shell coverage within a bed's boundaries. Two teams randomly survey portions of 60 DOQQs using shallow draft boats at or near MLW for verifi-

cation of the processed imagery. Field efforts also utilize Trimble Pathfinder Pro XR™ surveying units to measure reef length and position, as well as to note oyster density ('strata') and average reef width. Real-time Digital8™ video is recorded for each reef and is post-processed to determine estimates of percent vertical oyster coverage in defined time segments. Helicopter flights are also used for inaccessible areas. Post-processed imagery is validated by identifying beds that are correctly and incorrectly identified at a predefined accuracy level. This project will allow us to: (1) complete future evaluations by periodically re-flying and reassessing the state's shellfish beds; (2) provide both SCDNR and other interested users with detailed imagery available for a manifold of other uses; and (3) focus our oyster bed restoration efforts relative to current state management plans and status and trends analyses.

**APPROACHES TO SMALL-SCALE OYSTER RESTORATION: SITE CRITERIA AS A MEANS OF DETERMINING OPTIMAL METHODS FOR PUBLIC INVOLVEMENT.** Loren D. Coen<sup>1</sup>, Roh Brumbaugh<sup>2</sup> and Nancy H. Hadley<sup>1</sup>. <sup>1</sup>Marine Resources Research Institute, 217 Fort Johnson Rd., Charleston, SC 29912, SCDNR, <sup>2</sup>The Nature Conservancy, Global Marine Initiative.

*Crassostrea virginica* reefs were once a dominant feature of most Atlantic and Gulf coast estuaries, but have drastically declined in many areas across the U.S. Once valued primarily as a fishery resource, oysters are now recognized as key elements of many estuarine ecosystems, with a diverse set of attendant ecosystem services. Large- and small-scale restoration of subtidal and intertidal oyster habitats is ongoing in most Atlantic and Gulf coast states. For small-scale community-based restoration projects, an array of restoration approaches are in use, each of which has relative merits and limitations. Examples of these approaches include oyster gardening for stock enhancement, deployment of pre-filled oyster shell bags for intertidal restoration, and community-based shell recycling. We discuss the characteristics of these approaches, as well as several other deployment methods under consideration, and examine the logistical benefits and limitations of each. We also identify specific site criteria such as recruitment or substrate limitation that may be useful for identifying the most appropriate methodology for practitioners to employ in small-scale restoration projects.

**MOLECULAR GENETIC VARIATION AMONG QPX ISOLATES.** Jackie L. Collier, Hua Qian, Qianqian Liu and Bassem Allam. Discovery 145, Marine Sciences Research Center, Stony Brook University, Stony Brook, NY 11794-5000.

QPX has caused significant mortality of hard clams, *Merccenaria mercenaria*, in many locations along the east coast of North America since the 1960's, and a new outbreak of QPX occurred in

Raritan Bay NY during the summer of 2002. We have cultured new isolates of QPX from Raritan Bay clams collected in 2003 and 2004 and are using them, along with the Massachusetts isolate ATCC50749 and new Massachusetts QPX cultures isolated in 2005, to investigate the molecular genetic variability within QPX. We have found no sequence variability in the 18S and 5.8S rRNA genes from 6 New York isolates and ATCC50749. In contrast, the rRNA operon intergenic transcribed spacers, ITS1 and ITS2, showed a great deal of variability. Interestingly, the variation among ITS1 and ITS2 sequences from a single isolate was as great as the variability among the eight QPX isolates examined (seven from NY and ATCC50749). This result shows that the many copies of the rRNA operon in the QPX genome are not homogenized by gene conversion quickly enough to overcome the accumulation of sequence variation, and may suggest that QPX reproduces predominantly asexually. Sequences of 4 mitochondrial protein-coding genes also showed no sequence variability within or between the four isolates (two from NY and two from MA) examined. We will also present data from our current efforts to sequence non-coding regions of the mitochondrial genome.

**PERKINSUS MARINUS INFECTION IN OYSTERS FROM SOUTHEASTERN NORTH CAROLINA TIDAL CREEKS WITH VARYING WATER QUALITY.** Sara L. Colosimo, Martin H. Posey and Troy D. Alphin. University of North Carolina Wilmington—Center for Marine Science, 5600 Marvin K. Moss Lane, Wilmington, NC 28504.

On the East coast of the United States, oysters represent a critical fishery and impact many ecosystem functions, affecting water quality and providing habitat for a variety of fish and invertebrates. However, the Eastern oyster, *Crassostrea virginica*, has experienced major mortality throughout its range; attributed in part to disease caused by the protozoan parasite *Perkinsus marinus* (Dermo). This mortality has not been as severe for intertidal oyster populations in southeastern North Carolina as it has been in other areas. Anthropogenic stressors associated with storm water runoff, such as non-point source nutrient inputs and suspended particulates, may contribute to declining water quality and affect infection rates. Exposure to such environmental stressors can adversely affect oysters and increase their vulnerability to infection. This study compares *P. marinus* infection levels in oysters from three southeastern North Carolina tidal creeks that vary in water quality characteristics. Prevalence and intensity of *P. marinus* infection are being compared among creeks for both natural oyster populations and hatchery stock outplants using RFTM tissue assay. Data on growth, condition index, and mortality are also being compared. We predict that oysters in creeks with higher levels of nutrient inputs and particulates will have higher prevalence and intensity of infection. Preliminary results indicate that infection levels for all three creeks appear to be similar. For each creek, average infection intensity is light and infection is nearly 100% prevalent. Despite

low overall infection intensities, hatchery oysters in each creek have an average mortality of 20%.

#### CHARACTERIZATION OF BAC LIBRARIES FROM THE OYSTERS *CRASSOSTREA VIRGINICA* AND *C. GIGAS*.

Charles Cunningham<sup>1</sup>, Junichi Hikima<sup>1</sup>, Robert Chapman<sup>1</sup>, Matthew J. Jenny<sup>1</sup>, Jeff Tomkins<sup>2</sup> and Greg W. Warr<sup>1</sup>. <sup>1</sup>Hollings Marine Laboratory, Charleston, SC 29412. <sup>2</sup>Clemson University Genomics Institute, Clemson, SC 29634.

BAC libraries of two commercially and economically important species, *C. virginica* and *C. gigas*, have been developed as part of an international effort (co-ordinated by the Oyster Genome Consortium) to develop tools and reagents that will advance our ability to conduct genetic and genomic research on oysters. A total of 73,728 *C. gigas* clones with an average insert size of 150 kb were picked and arrayed representing approximately 12-fold genome coverage. A total of 55,296 clones with an average insert size of 134 kb were picked and arrayed for *C. virginica* representing approximately 10-fold coverage. The *C. gigas* and *C. virginica* libraries were screened with probes derived from 13 and 14 randomly selected genes respectively. All of the probes tested detected multiple clones ranging from 8–25 clones for *C. virginica* and 5–50 for *C. gigas*. These numbers suggest that there is no gross bias in either library and that some of the genes probed are members of multi-gene families. We have conducted a preliminary analysis of polymorphism represented in the two libraries using gene-specific primers for PCR amplification and nucleotide sequencing from selected BAC clones. Results suggest that the degree of sequence difference between alleles is highly variable (in a gene-specific manner) and that variability is, as would be predicted, concentrated in intronic regions.

#### DESIGN AND CHARACTERIZATION OF A MULTI-SPECIES OYSTER CDNA MICROARRAY.

Charlie Cunningham<sup>1</sup>, Matt Jenny<sup>2</sup>, Robert Chapman<sup>3</sup>, Gregory Warr<sup>1</sup>, Jonas Almeida<sup>4</sup>, Ann Chen<sup>4</sup>, Dave Mckillen<sup>4</sup> and Hal Trent<sup>4</sup>. <sup>1</sup>University of New Mexico. <sup>2</sup>Woods Hole Oceanographic Institute. <sup>3</sup>South Carolina Department of Natural Resources. <sup>4</sup>Medical University of South Carolina.

The eastern oyster, *Crassostrea virginica*, and the Pacific oyster, *C. gigas*, are important economic resources from both the harvesting of natural populations and aquaculture industry. In addition, because of their vital role in estuarine ecosystems oysters are a valuable model organism for environmental research. In an effort to increase our understanding of the effects of environmental pressures and disease on oyster physiology and health, an international collaboration (the Oyster Microarray Consortium) was initiated to construct a multi-species cDNA microarray consisting of expressed sequence tags from *C. virginica*, *C. gigas*, and the oyster pathogen *Perkinsus marinus*. The microarray consists of 4,460

clones from *C. virginica*, 2,320 clones from *C. gigas*, and 384 clones from the oyster pathogen, *P. marinus*. Functional annotation of the oyster clones was performed by comparison to the Gene Ontology database. As part of the initial characterization of this array, tissue specific signatures were identified by gene expression profiling both gill and hepatopancreas tissues from both species of oyster. Hepatopancreas gene expression profiles were also obtained from *C. ariakensis*, an oyster species not represented by clones on the array. The utility of the microarray for comparative studies between multiple oyster species was determined by the analysis of several hundred clones demonstrating cross-hybridization potential. Correlations between hybridization intensity and sequence homology of several hundred clones were used to estimate the level of sequence identity necessary for sufficient cross-hybridization. Results demonstrate the benefits in gene expression profiling by having multiple genes from both oyster species present on the array.

#### **EXPRESSION ANALYSIS OF CANDIDATE LOCI FOR GROWTH HETEROSIS IN THE PACIFIC OYSTER.** Jason Curole, University of Southern California.

The Pacific oyster has emerged as a model system to understand the genetic and physiological mechanisms of growth heterosis. A set of candidate loci for growth heterosis has been identified through comparative transcriptomic analysis of hybrid and inbred 6-day old oyster larvae. Evaluation of candidate loci requires discovery of polymorphisms, genetic mapping, and detailed analysis of expression patterns. *Crassostrea gigas* has a high density of single nucleotide polymorphisms and a surprisingly high density of indels (Curole & Hedgecock 2005, [http://www.intl-pag.org/13/abstracts/PAG13\\_W026.html](http://www.intl-pag.org/13/abstracts/PAG13_W026.html)). Using size fragment analysis to detect indel differences between parental alleles, I have genotyped eight of these candidates in a phenotyped  $F_2$  population. In addition, I have examined expression at several candidate loci in an  $F_1$  population and identified SNPs in this population for analysis of allele-specific expression.

#### **BIOENERGETICS OF JUVENILE PINK ABALONE FED FORMULATED DIETS CONTAINING DIFFERENT LEVELS OF PROTEIN AND STARCH: LIPID RATIOS.** Louis R. D'Abramo<sup>1</sup>, Jessica Montano-Vargas<sup>2</sup>, Armando Shimada<sup>3</sup>, Carlos-Vasquez-Pelaez<sup>3</sup> and Maria Teresa Viana<sup>2</sup>. <sup>1</sup>Department of Wildlife and Fisheries, Mississippi State University, <sup>2</sup>Universidad Autonoma de Baja California, <sup>3</sup>Universidad Nacional Autonoma de Mexico.

Juvenile pink abalone, *Haliotis corrugata*, (0.15 g) were grown for 131 days under laboratory conditions in aerated flow through seawater systems and fed semi-purified diets containing three different levels of protein, each level containing two ranges of starch lipid ratios (1.5 to 1.87 and 3.2 to 3.6), and similar gross energy

levels. Growth responses suggested a dietary protein requirement of approximately 35% with carbohydrate being the principal source of energy (high starch to lipid ratios). Optimum dietary lipid levels appear to be principally based on the satisfaction of specific nutrient requirements. Ammonia excretion ( $\mu\text{g NH}_4^+ \text{h}^{-1} \text{g}^{-1}$ ) ranged from 7.9 to 4.8 but was not significantly different among dietary treatments, except for the treatment containing 32% crude protein and a low carbohydrate: lipid ratio. Energy lost due to mucus production did not significantly differ among dietary treatments. Specific dynamic action comprised nearly 50% of the measured oxygen consumption ( $\mu\text{L O}_2 \text{h}^{-1} \text{g}^{-1}$ ) and did not differ significantly among dietary treatments. Respiration increased during the evening, suggesting a circadian pattern that is typical of the *Haliotis* genera. Approximately 70% of the ingested energy was lost to feces, and 25% metabolized, with 7 to 10% channeled to growth. A generalized pattern of utilization of dietary energy based upon collective investigations with *Haliotis* sp. is presented and suggests that 40–50% is generally lost to fecal production and approximately 50% of assimilated energy is lost to respiration.

#### **THE RELATIONSHIP BETWEEN PRODUCT QUALITY AND BIOCHEMICAL COMPOSITION IN SEA URCHIN GONADS, WITH EMPHASIS ON TASTE AND TEXTURE.** Trine Dale, Sten I Siikavuopio, Anders Aksnes, Brütt Hoppe and Mats Carlehög, Norwegian Institute of Fisheries and Aquaculture Research, Fiskerikorskning, 9291 Tormsø, Norway.

Whilst most sea urchin feeds at this point seems capable of promoting gonad growth, there is scope for improvement with respect to texture and taste. This study aims to identify possible common biochemical compounds associated with good (sweet, firm) and bad (bitter, melting) quality in sea urchins, and furthermore how compounds correlated with good quality is influenced by diet. The study is combining sensory analysis with analysis of proximate composition and free amino acids. In part one of the study, three species of wild sea urchins are compared: *Strongylocentrotus droebachiensis* (from Norway and Canada), *S. intermedius* and *Echinus esculentus*. In part two *S. droebachiensis* were fed five diets of different protein and carbohydrate content. In the wild sea urchins there was no apparent relationship between texture and proximate composition. There appear to be a relationship between the intensity of bitter taste and the content of valine and/or the content of alanine and glycine (increased bitterness with increased content of valine and/or reduced content of alanine and glycine). The intensity of sweet taste appears to be related to content of alanine and glycine. The sensory panel identified significant differences in quality between gonads from sea urchins fed different diets. The data from the biochemical analyses of these gonads are not yet analysed.



**GEODUCK AQUACULTURE IN NORTH AMERICA.****Jonathan P. Davis**, Taylor Resources, Inc. Quilcene, WA 98386.

Aquaculture development for geoduck clams (*Panopea abrupta*) increased rapidly over the last decade on the US and Canadian west coast. Intertidal and sub-tidal culture was initiated in the late 1980s by the Washington Department of Fisheries in an enhancement program to replace clams removed via commercial harvest. Though unsuccessful as an enhancement tool, rearing methods enabled aquaculture development to occur rapidly in Washington State and BC. Culture activities were initiated in the mid 1990s in both Washington State and BC, and more recently in Alaska and Oregon and expanded rapidly as more reliable methods for intertidal and sub-tidal grow out of geoducks were developed. Intertidal culture has been the focus in Washington where tidelands are either owned or leased, while sub-tidal plantings have been required in BC due mainly over restrictions to utilization of tidelands. Limitations on the growth of the industry are based mainly on seed availability and access to suitable intertidal sites. Though 4–6 years are necessary to produce a harvestable product, in excess of 200,000 KG are harvested annually with a landed value of US \$4.5 million currently with significant expansion in supply expected over the next decade. Developments in hatchery rearing of larvae and seed include a better understanding of nutritional requirements, the provision of live algal feeds high in poly-unsaturated fatty acids, and husbandry practices that focus on controlling clam density and pathogenic bacteria.

**DIGESTIVE ENZYME ACTIVITY IN BIVALVE TISSUES.****Lewis E. Deaton**, University of Louisiana at Lafayette, Lafayette, LA 70504.

While the major organ of digestion in bivalve molluscs is the digestive gland, there is direct and indirect evidence that the gills secrete some digestive enzymes. These enzymes may play a role in internal defense, but they may also be secreted to 'pre-digest' material that is to be swallowed by the animal. This latter notion is consistent with recent research demonstrating that at least in some species of bivalve, the sorting of material trapped on the gills into food to be swallowed and material to be rejected occurs on the gill itself. We have used API ZYM test kits (bioMérieux) to compare the activity of 19 assorted digestive enzymes in the gill, digestive gland, and adductor muscle of the quahog *Mercentaria mercenaria* and the oyster *Crassostrea virginica*. For most of the enzymes, the activity was highest in the digestive gland and lowest in the adductor muscle. Activities in the gill were intermediate between the former two tissues. Assays are being developed to examine the activity of laminarinase, cellulase, amylase, and proteases in gill, digestive gland and adductor muscle in *M. mercenaria* and *C. virginica*.

**SURVIVAL AND GROWTH OF *CRASSOSTREA VIRGINICA* LINES IN CHESAPEAKE AND DELAWARE BAYS.****Lionel Dégremont<sup>1</sup>, Standish K. Allen Jr.<sup>1</sup>, E. M. Burreson<sup>1</sup> and Gregory Debrosse<sup>2</sup>**, <sup>1</sup>Virginia Institute of Marine Science, Aquaculture Genetics and Breeding Technology Center, 1208 Greate Rd., Gloucester Point, VA 23062, <sup>2</sup>Haskin Shellfish Research Laboratory.

As a part of our oyster breeding program developed to enhance resistance against MSX and Dermo in *Crassostrea virginica*, eight lines and one control were produced in 2004 and deployed at four sites in Virginia (Burton's Bay, York River, Kinsale and Lynnhaven) and one site in the Delaware Bay (Cape Shore). First results, excluding the Cape Shore site, showed difference of mortality among sites in November 2005 with the highest mortality at Burton's Bay (57%), intermediate at the York River and Lynnhaven sites (40–46%) while oysters at Kinsale showed the lowest mortality (10%). The mortality mostly occurred between August and November except at Burton's Bay where it happened in April. Significant differences of mortality were also observed among lines in each site and a significant line by environment interaction was found which is easily explainable by the absence of significant mortality at Kinsale and the unusual mortality event occurring in April at the Burton's Bay. For growth, oysters deployed at York River and Lynnhaven showed the slowest growth while those at Kinsale and Burton's Bay showed the fastest. Finally, our results suggested which line would be most useful according to their yields across environments. For example in Burton's Bay, CROS-Breed line yielded 2.5 as much as the control while at Kinsale, a Louisiana strain would be preferred, yielding 60% greater than the control. Pathology analyses indicate MSX explains the mortalities observed during the summer and results from the fall sampling will provide more perspective on the cause of the mortalities.

**THE OTHER SIDE OF RESTORATION.** **Donatella Del Piero**, Viagrogrieri 10, Dipartimento di Bologna, Università Di Trieste, 34133 Trieste, Italy.

The tentative restoration done by the fishermen of shellfish beds (mainly *Chamelea gallina*) in Northern Adriatic seems to be far from optimization. Traces of uncontrolled restocking operations date back at least to 1990, but it doesn't seem to be decisive for stock enhancement. We have, instead, evidence of overall heavy depletion in stock and low growth rates, so the trade organisation are asking for diminishing the minimum legal size, actually set to 25 mm, till 20 mm, due to lack of legally marketable clams. This is a very old problem in the area and is of course questionable if the lack of response (e.g. to fleet reduction and long closure season) may reflect changes in the whole system or is an effect of gear improvement, bad knowledge of population dynamic, physiology etc. that must be known for implementing restoration programs.



**LAND-BASED SEA URCHIN CULTURE SYSTEM.** Michael Devin, University of Maine.

We are conducting a project to further develop and refine the culture conditions to optimize the growth of juvenile green sea urchins (*Strongylocentrotus droebachiensis*), a commercial species not previously cultured as juveniles. As part of this project we are investigating the effects of diet on growth from the early- to late-juvenile stages. To study growth we conducted an experiment whereby juvenile sea urchins were divided into seven treatments (*Ulva*, *Laminaria*, *Palmaria*, a mixed diet containing all three macroalgae, *Laminaria* augmented with mussel, *Palmaria* augmented with mussel and an artificial palletized formulation (Dr. Addison Lawrence formulation). Urchins were placed in colanders that contain a buoyant material so that they float with the rim approximately two cm above the surface. There were four replicates for each treatment. The animals were fed *ad libitum* twice weekly. Growth was highest in the mixed diet (mean wt. 26.0 gm), followed by the treatments of *Palmaria* (mean wt. 23.6 gm) and the artificial formulation (mean wt. 23.6 gm). Higher growth rates found in treatments containing *Palmaria* are postulated to be due to the high percentage of protein and calcium found in *Palmaria* compared with *Ulva* and *Laminaria*. Although, juvenile sea urchins appear to grow fastest when fed *Palmaria* relative to *Ulva* and *Laminaria*, the mixed treatment produced the largest animals.

**THE EFFECTS OF DIET ON GROWTH OF THE GREEN SEA URCHIN (*STRONGYLOCENTROTUS DROEBACHIENSIS*) FROM THE EARLY- TO LATE-JUVENILE STAGES.** Michael G. Devin, Henry D. Stence, and Robert J. Peacock. R. J. Peacock Canning Company, 72 Water Street, Lubec, Maine 04652.

We are conducting a project to further develop and refine the culture conditions to optimize the growth of juvenile green sea urchins (*Strongylocentrotus droebachiensis*), a commercial species not previously cultured as juveniles. As part of this project we are investigating the effects of diet on growth from the early- to late-juvenile stages. To study growth we conducted an experiment whereby juvenile sea urchins were divided into seven treatments (*Ulva*, *Laminaria*, *Palmaria*, a mixed diet containing all three macroalgae, *Laminaria* augmented with mussel, *Palmaria* augmented with mussel and an artificial palletized formulation (Dr. Addison Lawrence formulation). Urchins were placed in colanders that contain a buoyant material so that they float with the rim approximately two cm above the surface. There were four replicates for each treatment. The animals were fed *ad libitum* twice weekly. Growth was highest in the mixed diet (mean wt. 26.0 gm), followed by the treatments of *Palmaria* (mean wt. 23.6 gm) and the artificial formulation (mean wt. 23.6 gm). Higher growth rates found in treatments containing *Palmaria* are postulated to be due to the high percentage of protein and calcium found in *Palmaria* compared with *Ulva* and *Laminaria*. Although, juvenile sea ur-

chins appear to grow fastest when fed *Palmaria* relative to *Ulva* and *Laminaria*, the mixed treatment produced the largest animals.

**SHELLFISH CROP AND CULTURE GEAR FOULING IN THE PACIFIC NORTHWEST.** Bill Dewey, Taylor Shellfish Company, 130 SE Lynch Rd., Shelton, WA 98584.

Shellfish culture in the United States Pacific Northwest like many areas of the world is complicated by biofouling of shellfish crops and aquaculture gear. Growers in this part of the country are plagued by such things as barnacles, overcatch of oysters on single oyster crops, suffocating macroalgae as well as *Spartina* and eelgrass infiltration. Subtidally, crops and gear are fouled by barnacles, bryozoans, sponges and tunicates. Recently the discovery of non-native colonial tunicates (*Didemnum* sp.) in Puget Sound has subtidal shellfish growers concerned. Depending on circumstances and severity biofouling can have detrimental effects on growth and survival of crops. Each geographic farm location typically has its own unique fouling issues. Fouling will vary geographically, annually and seasonally. In the case of intertidal culture, which is the primary means of culturing in the Pacific Northwest, fouling will also vary with tidal elevation. Assorted husbandry or culture strategies are employed to minimize and/or avoid fouling or to clean fouling organisms off gear and crops.

**RESTORING *MERCENARIA* ON LONG ISLAND: CONDITION AND SPAWNING OF TRANSPLANTED HARD CLAMS IN SANCTUARIES IN LONG ISLAND, NEW YORK.** Michael H. Doall<sup>1</sup>, Dianna K. Padilla<sup>1</sup>, Carl Lobue<sup>2</sup> and Laurie Perino<sup>1</sup>. <sup>1</sup>Stony Brook University, Department of Ecology and Evolution, Stony Brooke, NY 11794-5245. <sup>2</sup>The Nature Conservancy.

On Long Island, New York, the Nature Conservancy has spearheaded efforts to restore *Mercuraria mercenaria* populations by creating sanctuaries of high densities of spawning clams to reduce sperm limitation for fertilization and thereby increase the potential for recruitment. Success requires that the clams survive, obtain enough energy for gonad development, and spawn. To assess the condition of transplanted clams in sanctuaries established in the Great South Bay and Peconic estuaries, we monitored condition index (CI), computed as the ratio of the dry mass of soft tissue to the internal shell cavity capacity, and qualitative rankings of gonad ripeness during 2004 and 2005. Changes in CI were correlated with changes in gonad ripeness in all sanctuaries monitored; CI increased as gonads ripened and decreased as gonad tissue and gamete concentrations diminished. Decreases in CI and gonad ripeness coincided with the appearance of hard clam larvae in the plankton, indicating that spawning had occurred. The magnitude and timing of changes in CI and gonad ripeness varied between populations of clams in different sanctuary sites, and between years for the same populations. Temperature appeared to be one

exogenous factor influencing the timing of reproductive cycles, but spatial-temporal variability in other environmental parameters, such as food quantity and quality, may also explain differences between populations and between years. The prior history of transplanted clams, especially their source location, also appeared to influence condition and timing of gonad development during the first year after transplant.

**GEODUCK FARMING 101—THE TRIALS AND TRIBULATIONS OF STARTING A NEW FARM.** Peter Downey, Discovery Bay Shellfish Inc.

This presentation will examine issues encountered while planting three acres of geoduck during the 2005 planting season. Issues to be reviewed include securing appropriate tidelands, coming to terms with regulatory agencies and tribes, developing a labor pool, ensuring seed survival, planting logistics and dealing with inclement weather and wind driven waves.

**HUSBANDRY PRACTICES AND ASSOCIATIONS WITH PRODUCTIVITY IN PRINCE EDWARD ISLAND MUSSEL FARMS.** Andre Drapeau<sup>1</sup>, Luc Comeau<sup>2</sup> and Jeff Davidson<sup>1</sup>. <sup>1</sup>University of Prince Edward Island, <sup>2</sup>Department of Fisheries and Oceans.

Associations between husbandry practices and blue mussel (*Mytilus edulis*) productivity in Prince Edward Island (PEI), Canada farms were investigated. Husbandry was measured using SCUBA in Tracadie Bay (2002–2004) and in 16 embayments in 2003. Measurements related to 1-year-old crop exposed to approximately eight months of husbandry practices. Between 2002 and 2004, longline spacing, sock spacing and sock weight in Tracadie Bay ranged from 5 to 24.85 m, 27.50 to 40.40 cm and 2.86 to 15.49 kg respectively, while across PEI husbandry ranged from 1.50 to 29.54 m, 26.38 to 62.40 cm and 1.48 to 16.87 kg respectively. Sock spacing significantly increased by 32% (+12 cm) over the years and was directly responsible for significantly decreasing lease stocking density by 25% (–6 socks/100 m<sup>2</sup>). Regression models revealed that sock spacing was positively associated with mussel sock weight in Tracadie Bay (2002) and across PEI (2003). In Tracadie Bay, sock spacing was related to sock weight in 2002 and mussel condition in 2004. Each additional 10 cm between socks resulted in raising predicted sock weight by 1.15 kg and mussel condition index by 1.60, while across PEI, sock weight increased 0.74 kg. This gain is plausible and likely driven by reduction in intraspecific competition between mussels for available food resources. However, the observed correlation was statistically significant only for a single year in Tracadie Bay (2002), while marginally significant across PEI (2003). This suggests that the effect of sock spacing was dynamic and dependent upon food availability within the water column.

**OYSTER SURVIVAL AND GROWTH IN THE BARATARIA ESTUARY.** Ronnie Duke and Earl J. Melancon, Department of Biological Sciences, Nicholls State University, P.O. 1, Thibodaux, LA 70310.

We documented oyster responses to natural environmental events in the historically productive zone of the Barataria estuary, part of the Davis Pond Mississippi River Diversion outfall area. The diversion has operated minimally with no measurable impact on oysters. During the study there were two naturally occurring reduced-salinity events (<5 ppt), two tropical storms and three hurricanes.

Oyster populations were studied monthly from April 2004 to November 2005 for survival, size frequency and condition index. In June/July 2004 salinities were well below 5ppt across most of the estuary and all oysters died except at the most down-estuary site. Oysters rebounded with a good estuary-wide spat set in September/October 2004. All fall 2004 set oysters survived an estuary-wide winter 2005 reduced-salinity event. Oysters exhibited highest condition indices during winter and lowest during summer.

Seed-sized oysters, 44 mm in shell length, were tagged in fall 2004 at two sites for monthly growth comparisons. From September 2004 to August 2005 oysters grew to  $64.3 \pm 6.9$  mm at the up-estuary site and to  $77.5 \pm 7.3$  mm at the down-estuary site. A shell length of 65 mm is minimum sack size acceptable for oysters harvested from private leases. Preliminary comparisons of the fall 2004 sets at 10 sites for size frequency exhibited a similar pattern of faster growth down-estuary.

This information becomes important for natural resource managers and oystermen as adaptive management strategies are developed so that both diversions and oysters can co-exist. Oystermen must decide if they want to harvest seed-size oysters or wait the additional time to harvest sack-sized oysters.

**OYSTER AQUACULTURE IN A PACIFIC NORTHWEST (USA) ESTUARY: A PLEA FOR TAKING A LANDSCAPE-ECOSYSTEM PERSPECTIVE.** Brett R. Dumbauld<sup>1</sup>, Jennifer Ruesink<sup>2</sup>, and Miranda Wecker<sup>3</sup>. <sup>1</sup>U.S. Department of Agriculture, Agricultural Research Service, 2030 SE Marine Science Dr., Newport, OR 97365, <sup>2</sup>University of Washington, Department of Biology, WA 98195, <sup>3</sup>University of Washington, Olympic Natural Resources Center, Forks, WA 98331.

Commercial shellfish harvest in Willapa Bay, Washington has been an important source of oysters for 150 years and aquaculture in this estuary alone produces about 10% of the U.S. oyster harvest. Management policies for estuarine tidelands in the Pacific Northwest are based on general descriptors and perceptions from research conducted elsewhere (e.g. submerged aquatic vegetation provides structure which increases abundance and diversity of other organisms), resulting in regulations such as no-net loss of aquatic vegetation which are applied on a very small individual permit scale. While results of recent studies conducted at this scale

in Willapa Bay and other Pacific coast estuaries do in-part confirm the generality of these descriptors, these estuaries appear to function slightly differently and the role of cultured oysters as habitat and as secondary consumers in these systems is newly described. Scale is clearly an issue and recent mapping efforts in Willapa Bay suggest that managers should view aquaculture from an estuarine landscape perspective. Techniques that have recently been applied for analyzing the role of terrestrial agriculture in otherwise forested or grassland ecosystems suggest that movement corridors and edge-interior ratios are important considerations. Preliminary results from such an exercise conducted in Willapa Bay suggest that traditional policies which seek to conserve other large invertebrate or vertebrate fisheries by protecting one habitat (aquatic vegetation) may need to be reconsidered once results are confirmed by directed studies conducted at this landscape scale. Considering local differences, best management practices could then also be regulated and implemented at this scale.

**PERKINSUS OLSENI IN VITRO ISOLATES FROM NEW ZEALAND CLAMS (*AUSTROVENUS STUTCHBURYI*).**

Christopher F. Dungan<sup>1</sup>, Rosalee M. Hamilton<sup>1</sup>, Kimberly S. Reece<sup>2</sup>, Jessica A. Moss<sup>2</sup>, and Ben K. Diggles<sup>3</sup>. <sup>1</sup>Maryland Dept. of Natural Resources, Oxford Laboratory, <sup>2</sup>Virginia Institute of Marine Science, <sup>3</sup>DigsFish Services Pty Ltd, Queensland, Australia.

Prevalent *Perkinsus* sp. infections have been reported in *Austrovenus stutchburyi* venerid clams (cockles) from northern New Zealand, since 2000. Sequencing of rRNA gene complex ITS- and NTS-regions of genus-*Perkinsus* PCR amplicons from infected *A. stutchburyi* DNA templates, have consistently indicated the presence of *P. olseni* parasites. However, *in vitro* *Perkinsus* sp. isolates have been unavailable to date for comprehensive morphological and genetic evaluation of the New Zealand parasite's identity. We report *in vitro* isolation, propagation, cloning, and cryopreservation of four *Perkinsus* sp. isolates from a 2005 sample of *A. stutchburyi* cockles from northern New Zealand's Mangamangaroa Stream (24°C, 32 ppt). Isolates were propagated *in vitro* despite a moderate prevalence (24%) of low-intensity *Perkinsus* sp. infections in the sampled cockles. These isolates showed morphometric similarities and differences with characteristics reported for *P. olseni* isolates from other hosts and locations. Although their frequency of continuous *in vitro* zoosporulation was low, one typical isolate was induced to wholesale (>70%) zoosporulation by a transient, 48-h passage through ARFTM. Nucleotide sequences of rDNA ITS-regions from our isolates consistently grouped them with *P. olseni* sequences in phylogenetic analyses, suggesting that *Perkinsus* sp. infecting New Zealand *A. stutchburyi* clams are conspecifics of destructive and broadly distributed *P. olseni* parasites of several eastern Asia clams, diverse Australian clams and abalone, and several European clams.

**WASHINGTON STATE GEODUCK AQUACULTURE PROGRAM.** Sarah Dzinbal, Celia Barton and Jeffrey Schreck. Washington State Department of Natural Resources, Aquatic Resources, P.O. Box 47027, Olympia, WA 98504-7027.

The 2003 Washington State Legislature directed the Washington State Department of Natural Resources (DNR) to determine the feasibility of geoduck aquaculture on state owned aquatic lands (SOAL). Between July 2003 and June 2005, DNR contracted with the University of Washington and other entities to compile reports that would allow that determination to be made. DNR testified to the 2005 Legislature that geoduck aquaculture on SOAL is feasible, but recommended that the project proceed with caution by authorizing the use on twenty to twenty-five acres of SOAL per year. DNR submitted a budget request for additional funds to implement a leasing program for geoduck aquaculture on SOAL, but unfortunately did not receive those funds. DNR believes that aquaculture is an important use and, as such, has moved forward with implementation of a geoduck aquaculture-leasing program. The lack of dedicated funding has necessitated a different approach—DNR is designing a monitoring program that will require some of its geoduck aquaculture lessees conduct scientific monitoring on their leaseholds throughout the first geoduck growing cycle. The results of this monitoring will allow DNR to fully assess the environmental effects of geoduck aquaculture, and further allow a determination on the long-term feasibility of this use. Twenty to twenty-five acres of SOAL will be put up for competitive bid in the summer of 2006. Potential lessees will respond to the Request for Proposals by providing a summary of their proposed activities, including the scientific monitoring aspect.

**WASHINGTON STATE GEODUCK FISHERY MANAGEMENT.** Sarah Dzinbal, Celia Barton and Todd Palzer. Washington State Department of Natural Resources, Aquatic Resources, P.O. Box 47027, Olympia, WA 98504-7027.

The Washington State Department of Natural Resources (DNR), as the steward of publicly owned lands, manages approximately 2.4 million acres of aquatic lands in Washington. One of its roles is to co-manage the wild stock geoduck fishery, and the agency does this together with the Washington Department of Fish and Wildlife (WDFW) and the Treaty Tribes in western Washington. The geoduck fishery has been open since 1970 and is managed to be sustainable for future generations of citizens in Washington State. The state and tribes work collaboratively to quantify geoduck biomass by conducting tract surveys on a rotational basis. WDFW sets the Total Allowable Catch for each fishery year—2.7% of the total commercial geoduck biomass—and the state and tribes share this resource equally. DNR publicly auctions the right to harvest the state's share of the geoduck resource, in the form of geoduck quotas. This generates approximately \$8 million per year for the state, which is used by a number of state and local government entities for aquatic land enhancement and management

work. The successful bidders at auction sign harvest agreements with DNR; the agreements allow companies to harvest specified pounds of geoducks from specified tracts of state owned aquatic lands for a specified period of time. The agreements are enforced by DNR's commercial dive team, which conducts daily fishery compliance operations on the geoduck tract every day the state fishery is open. The fishery is a year-round dive fishery.

**USE OF THE OLIGOHALINE CLAM *RANGIA CUNEATA* AS A BIVALVE INDICATOR OF WATERSHED ALTERATIONS IN SOUTHWEST FLORIDA.** Vincent Encomio<sup>1</sup>, Christina Panko<sup>1</sup>, Ernest Estevez<sup>2</sup> and Aswani K. Volety<sup>1</sup>.

<sup>1</sup>Coastal Watershed Institute—Florida Gulf Coast University, 10501 FGCU Blvd S., Fort Myers, FL 33965, <sup>2</sup>Center for Coastal Ecology—Mote Marine Laboratory Sarasota, Sarasota, FL.

In Florida, freshwater releases from Lake Okeechobee result in dramatically lowered salinity, adversely affecting bivalve populations downstream in the Caloosahatchee River. Additionally, high sediment loading due to increased freshwater flow or dredging may adversely affect bivalve condition. The impact of watershed alterations on oysters is part of ongoing research in SW Florida. The use of oysters as a sentinel organism, however, only covers the mesohaline (10–30 parts per thousand (ppt)) regions of the estuary. Few, if any studies have examined effects on bivalves in the oligohaline (<10 ppt) portions of the Caloosahatchee estuary. The clam *Rangia cuneata* is found extensively in oligohaline regions of the Caloosahatchee and may be a useful bivalve indicator of sedimentation and altered salinity. Clams were exposed for thirty days to clay (<4 µM grain size) and silt (40–60 µM) at 0, 1 and 2 grams/clam/day. At 0, 10, 20 and 30 days condition index (dry tissue weight: dry shell weight), glycogen, lipid and protein content in clams were measured. Condition index decreased significantly over time ( $p = 0.0177$ ) but not with sediment type and dose. Despite wide physiological salinity tolerances, *R. cuneata* cannot maintain stable populations beyond 1–15 ppt (Hopkins et al. 1973), making it vulnerable to extended freshets and droughts. Distributions of live and dead shells indicate higher proportions of dead shells near the head of the estuary, possibly indicative of past freshets. Salinity tolerances and responses to interactive stress (e.g. temperature) will be examined in the lab to corroborate patterns seen in the wild.

**COMMUNITY SHIFT ASSOCIATED WITH SHELLFISH AQUACULTURE IN TWO MID-ATLANTIC ESTUARIES.**

Patrick Erbland and Gulnihal Ozbay, Delaware State University.

Populations of the eastern oyster (*Crassostrea virginica*) and its associated fishery have been declining in the State of Delaware and greater Mid-Atlantic region of the United States for decades.

This shortage of oysters continues despite intensive husbandry of oyster reefs by fishers and government agencies. Consequently there is interest in the culture of *C. virginica* enclosed in "grow out gear" (GOG) to increase yields. Such a practice has a multitude of associated impacts on the host ecosystems. These include reduced nutrient load in the water column (Ulanowicz and Tuttle 1992) and enrichment of benthic sediments (Nugues et al 1996). The enhanced levels of sport fish such as Striped Bass (*Morone saxatilis*) and Bluefish (*Pomatomus saltatrix*) found on oyster reefs (Harding and Mann 1998) may also exist on oyster farms. This is a two part study investigating shifts in the benthic and infaunal communities. We will compare the diversity and abundance of species inhabiting subtidal "Rack and Bag" type GOG, containing *C. virginica*, with an adjacent, created *C. virginica* reef in Indian River Bay, DE. Secondly, we will compare the diversity and abundance of infaunal species present below intertidal oyster gear with an adjacent control area of open sand flat on the eastern shore of Delaware Bay. This study will provide insight into the ecological impact of shellfish aquaculture and be useful in incorporation of *C. virginica* aquaculture into the management schemes of concerned regulatory agencies.

**ADDITIONAL EVIDENCE OF HIGH RESISTANCE TO *HAPLOSPORIDIUM NELSONI* (MSX) IN THE NATIVE OYSTER POPULATION OF DELAWARE BAY.** Susan Ford and David Bushek, Haskin Shellfish Research Laboratory, Rutgers, The State University of New Jersey, 6959 Miller Avenue, Port Norris, NJ 08348 USA.

Natural selection during the first *Haplosporidium nelsoni*-caused epizootic in Delaware Bay oysters resulted in the development of a degree of resistance to mortality caused by the pathogen throughout the Bay. Until the late 1980s, this level of resistance did not change because most of the surviving oysters were protected from additional selection as they resided in the low salinity region of the Bay. The development of a second level of resistance was signaled after heavy *H. nelsoni*-caused mortalities in the mid 1980s occurred during a severe drought and prevalence of the pathogen subsequently declined markedly. Susceptible controls exposed beside natural set in the lower bay continued to become heavily infected, whereas the natural set had prevalences of 0 to 10%. To determine whether oysters on the low-salinity beds were similarly resistant, we exposed oysters from two upper-bay beds along with lower bay natives and susceptible controls in Cape May Harbor where *H. nelsoni* pressure has been heavy. Six months later, prevalence of susceptible oysters was 90%, with most infections being advanced. At the same time, prevalence in the two upper bay groups was only 5 to 20% with mostly light, localized infections. This confirms that a high degree of resistance is now present throughout the Bay. In contrast, there is little evidence that

significant resistance to infection by *Perkinsus marinus* has developed despite continuous exposure and consequent mortality over the past 15 years.

#### COMPARATIVE EFFECTS OF NEUROTRANSMITTERS AND CALCIUM BLOCKERS ON ISOLATED GILLS AND WHOLE ANIMALS OF THE MUSSEL, *MYTILUS EDULIS*.

Dana M. Frank, J. Evan Ward, Sandra E. Shumway<sup>1</sup> and Lewis Deaton<sup>2</sup>. <sup>1</sup>University of Connecticut, 1080 Shennecossett Rd., Groton, CT 06340, <sup>2</sup>University of Louisiana at Lafayette.

It is generally accepted that the lateral cilia of bivalve molluscs are inhibited by dopaminergic systems and excited by serotonergic systems. Most of this experimentation has been carried out on excised gills of the mussel, *Mytilus edulis* and not on whole animals. We conducted preliminary tests to compare the effects of the neurotransmitters serotonin (5 HT), dopamine (DA), and apomorphine and the calcium channel blocker lanthanum on pumping activity of intact *M. edulis* and the ciliary activity of isolated gill tissue. We also exposed isolated gill filaments to the calcium channel blockers cobalt chloride and verapamil. Whole animals and freshly excised gill sections were kept in filtered seawater at ambient temperature (~20°C). Experimental drug was added directly to the bath water. Activity of the lateral cilia of isolated gills was monitored with an inverted compound microscope. Water processing rates of whole animals were examined using particle image velocimetry (PIV). Lanthanum induced shedding of the gill epithelia in excised sections and in whole animals obviating any effect on the lateral cilia. Cobalt chloride, DA, and verapamil had no effect on gill sections. When exposed to  $1 \times 10^{-6}$  M apomorphine, 30% of gill sections showed some inhibition; 50% were inhibited at  $1 \times 10^{-5}$  M. In the whole animal,  $1 \times 10^{-6}$  M apomorphine had a significant negative effect on velocities of particles in the excurrent flow. 5 HT appeared to increase synchrony (metachronism) of the lateral ciliary beat in isolated gill sections. Preliminary work suggests that neural agents affect whole animals differently than isolated gill tissue.

#### OPTIMIZATION OF OXYTETRACYCLINE TREATMENT IN TWO ABALONE SPECIES, *HALIOTIS SORENSENI* AND *H. RUFESCENS*.

Carolyn S. Friedman<sup>1</sup>, B. B. Scott<sup>1</sup>, R. E. Strenge<sup>1</sup>, N. A. Wight<sup>1</sup>, Thomas B. McCormick<sup>2</sup> and George Trevelyan<sup>3</sup>. <sup>1</sup>School of Aquatic and Fisheries Sciences, University Washington, Box 355020, Seattle, WA 98195, <sup>2</sup>Channel Islands Marine Research Institute, Port Hueneme, CA, <sup>3</sup>The Abalone Farm, Inc., Cayucos, CA.

Withering syndrome (WS), a rickettsial disease, causes losses of wild and farmed abalone. The endangered white abalone, *Haliotis sorenseni*, is highly susceptible to WS and information on management of this disease is essential for successful restoration. White abalone were fed a medicated diet containing oxytetracy-

cline (OTC) for 20 d; digestive gland (DG) and foot muscle (FM) were assayed for OTC and rickettsia at days 3, 18, 24, 40, 67, 80, 110, 129, 165 and 185 post-medication. Abalone were rechallenged with rickettsia at 24, 40, 67, 146 and 171 d. DG contained higher levels and retained OTC longer than FM. Mean DG drug levels peaked at 996 ppm at 25 d post-medication and leveled off at 30 ppm after 68 d. FM peaked at 18 ppm and declined to below 2 ppm, the FDA tolerance level, between 25 and 41 d. Protection from rickettsial infections occurred in abalone with over 50 ppm in the DG. We investigated the pharmacokinetics of one oral OTC dose in red abalone and that of 1, 2 or 3 intramuscular (IM) injections with Liquamycin-LA 200 in white abalone. One oral dose cleared 90–100% ( $p < 0.05$ ) of infections. A mean of 239 ppm OTC was detected in the DG 5 d after medication followed by depletion to 2.34 ppm after 130 d. In a second trial, 688 ppm accumulated in the DG at 1 d and declined to 326 ppm by day 5. Interestingly, total body OTC was only 89 ppm at day 1 (688 ppm in DG). IM injections resulted in DG accumulations of 7.15, 20.3 and 27.4 ppm after 1, 2 and 3 doses, respectively.

#### DEVELOPMENT AND APPLICATION OF TYPE I MARKERS FOR LINKAGE MAPPING AND POPULATION GENETICS IN *CRASSOSTREA* SPECIES.

Patrick Gaffney<sup>1</sup>, Hyungtaek Jung<sup>1</sup>, Woo-jin Kim<sup>2</sup>, Robin Varney<sup>1</sup> and Coren Milbury<sup>1</sup>. <sup>1</sup>University of Delaware, College of Marine Studies, 700 Pilottown Rd., Lewes, DE 19958, <sup>2</sup>National Fisheries Research & Development Institute.

In oysters, nuclear markers developed for linkage mapping and the analysis of population structure include allozymes, anonymous single copy DNA (scnDNA) loci, microsatellites, and most recently, single nucleotide polymorphisms (SNPs). The emergence of substantial expressed sequence tag (EST) databases for *Crassostrea virginica* and *C. gigas* has enabled researchers to probe candidate genes for polymorphisms by direct DNA sequencing. Because the number and locations of introns in genes selected from EST databases are unknown, primer design for amplification from genomic DNA can be challenging. Taking advantage of the reduced frequency of introns in the 3'UTR of invertebrate genes, we have designed primers for small (~300 bp) amplicons spanning the 3' end of coding sequence and the 3'UTR. These primers typically provide successful amplifications and often amplify putatively homologous sites in closely related *Crassostrea* species. High levels of polymorphism ensure that even short amplicons usually have one or more SNPs suitable for genotyping. Applications of markers developed to date will be discussed, including linkage mapping in *C. virginica* and *C. gigas*, population genetics and taxonomy of Atlantic and Asian oysters, and monitoring restoration efforts involving hatchery-produced seed.

**REPRODUCTIVE TIMING AND SUCCESS OF FRESHWATER MUSSELS.** Heather S. Galbraith, Caryn C. Vaughn and Daniel E. Spooner. Oklahoma Biological Survey and the University of Oklahoma.

Freshwater mussels are among the most imperiled groups of aquatic organisms on earth. Their diversity, dominance in benthic biomass, and unique life histories make them important contributors to stream ecosystem function while at the same time have made them susceptible to anthropogenic interferences such as river regulation, impoundments, and global warming. High species richness and large numbers of closely related species within mussel beds has proven paradoxical to typical views of speciation in broadcast spawning organisms. The goal of our research is to determine the reproductive timing, success, and isolation of three closely related species of freshwater mussels by addressing the following questions: 1) What are the mechanisms of reproductive isolation in freshwater mussels? 2) How is reproductive timing regulated and important to fertilization success in freshwater mussels? In order to conserve mussel communities, understanding the processes of speciation and reproduction are vital. This work will be some of the first to provide an evolutionary and ecological perspective on one of the most critical and little understood periods of the freshwater mussel life cycle, specifically that prior to fertilization.

**GREEN CRABS (*CARCINUS MAENAS*) AS THE GRIM REAPER: DESTRUCTION OF EELGRASS BEDS IN NOVA SCOTIA.** David J. Garbary and A. G. Miller. Department of Biology, St. Francis Xavier University, Antigonish, Nova Scotia, Canada B2G 2W5.

Following the first appearance of invasive *Carcinus maenas* in the southern Gulf of St. Lawrence in the early 1990s, 385,000 crabs km<sup>-2</sup> occurred in Antigonish Harbour in 2000. Eelgrass (*Zostera marina*) which had large stable populations in the estuary for the previous 60 years declined by 95% in 2001, and many parts of the harbour were devoid of living eelgrass. This decline also occurred in adjoining estuaries where large numbers of crabs also occurred, but did not take place in estuaries further west and north where green crabs had not yet invaded, or numbers were low. In July-August 2002, shoot density of eelgrass at an experimental site in Tracadie Harbour with high crab numbers declined by 75% when a control harbour with few crabs showed increasing shoot density. A field experiment using 1.5 m<sup>2</sup> cages, with and without green crabs, suggested that *C. maenas* was responsible for the loss of eelgrass. Telephone interviews with knowledgeable observers around Nova Scotia also suggested a correlation between declining eelgrass populations and increasing numbers of crabs during the previous ten years. The impact of green crabs on eelgrass is associated with their foraging activity in which they produce pits up to

30 cm wide and 15 cm deep. During foraging, eelgrass shoots are dislodged by crab digging, or the shoot bases are shredded, causing release of whole shoots into the drift. Since 2001, green crab numbers have been in decline in Antigonish Harbour, and there has been a substantial recovery of *Z. marina*.

**SYNTHESIS OF 15 YEARS RECORDS FOR *HAPLOSPORIDIUM NELSONI* IN *CRASSOSTREA GIGAS* IN FRANCE.** Céline Garcia, Isabelle Arzul, Bruno Chollet, Sylvie Ferrand, Cyrille François, Jean-Pierre Joly, Laurence Miossec and Maeva Robert

Haplosporidian parasites have been described in several bivalves such as oysters. The species *Haplosporidium nelsoni* is particularly well known in the American oyster *Crassostrea virginica* in which it induces massive mortalities. *H. nelsoni* was also detected in Pacific oyster *C. gigas* from different parts of the world. In France, it was detected for the first time in 1993 but other haplosporidian parasites had already been observed before in *C. gigas*.

The data analysis of the French network for surveillance of mollusk health between 1990 and 2004 showed a very sporadic detection of *H. nelsoni* (histology and confirmation by *in situ* hybridization); its detection frequency is of 1‰ per year on average and can reach 1% in a given area. It was mainly detected in oyster spat and in summer period (from May to August). It was essentially present along French Atlantic coasts, particularly in estuaries or in sheltered bays. In 2005, its detection frequency increased (1‰ to 1%) and reached 7 to 10% in some areas. As previously recorded, the parasite was observed in connective tissue from different organs and also in the epithelia of digestive diverticulae where spores were observed. It is the first time that a sporulation of *H. nelsoni* in *C. gigas* is described in France. This observation and the increase of its detection can raise some questions: does the parasite behaviour in Pacific oyster change? Does it adapt to the French environmental conditions? Does it present a risk for the French oyster culture?

**DETECTION AND DISTRIBUTION OF QUAHOG PARASITE UNKNOWN IN THE COASTAL MARINE ENVIRONMENT.** R. J. Gast<sup>1</sup>, D. M. Moran<sup>1</sup>, K. R. Uhlinger<sup>2</sup>, D. R. Leavitt<sup>3</sup> and R. Smolowitz<sup>2</sup>. <sup>1</sup>Woods Hole Oceanographic Institution, Woods Hole, MA 02543, <sup>2</sup>Marine Biological Laboratory, Woods Hole, MA, <sup>3</sup>Roger Williams University.

Quahog Parasite Unknown (QPX) is a significant cause of hard clam mortality along the Northeast coast of the United States. The QPX organism is classified as a thraustochytrid protist, and members of this protistan family are generally saprophytic and com-

monly found in the marine environment. No viable method was available to reliably survey the natural environment for QPX, to rapidly assess the persistence of the organism in previously affected plots, and to screen large numbers of clams potentially exposed to the disease. Here we report on a PCR-based method that we have developed that permits sensitive detection of QPX in natural samples and seed clams. With our method, between 10–100 QPX cells can be detected in 1 L of water, 1 gram of sediment and 100 mg of clam tissue. We have used the method to examine one hundred 15 mm seed clams, and found that 10–12% of the clams were positive for the presence of the QPX organism, although only 1% showed histological evidence of infection. The method has also been used in a survey for the presence of the pathogen in environmental samples from a site experiencing severe infection and a site with low to no infection. This survey has revealed the presence and year-round persistence of QPX in the environment, even in the absence of large-scale die-off.

**CONDITION OF FRESHWATER MUSSELS HELD IN REFUGIA AT THE WHITE SULPHUR SPRINGS NATIONAL FISH HATCHERY, WEST VIRGINIA.** Catherine M. Gatenby<sup>1</sup>, Matthew A. Patterson<sup>1</sup>, Julie L. Devers<sup>1</sup> and Danielle A. Kreeger<sup>2</sup>. <sup>1</sup>United States Fish and Wildlife Service, <sup>2</sup>Delaware River Basin Commission.

The USFWS established a refugium at the WSSNFH for endangered freshwater mussels residing in harms way of a bridge replacement in the Allegheny River. Approximately, 600 common mussels from two species sharing a similar reproductive strategy as target endangered species were salvaged in October, 2004; endangered mussels were salvaged in May 2005 as well. In August 2005, 450 common mussels were relocated to WSSNFH, and were included in a diet quality feeding study. Non-lethal and lethal measures of physiological activity and condition are used to monitor condition of common mussels.

After one year in captivity, short-term brooders have maintained condition and survival better than long-term brooders. Condition of *Elliptio dilatata* was not significantly different than wild *E. dilatata* in September 2005; whereas, captive *Actinonaias ligamentina* condition was significantly less than their wild counterparts. Endangered *Pleurobema clava* had higher survival (100%) than endangered *Epioblasma t. rangiana* (8%). Similarly, *E. dilatata* (74%) had higher survival than *A. ligamentina* (66%). Mussels collected in October 2004 were unusually low in physiological condition, and were likely stressed by high water conditions and high suspended bed loads in the Allegheny River throughout most of 2003 and 2004. WSSNFH modified culture systems in May 2005 to improve food delivery and food quality. Survival of endangered mussels brought into captivity in spring 2005 ranges 93%–100% for *E. t. rangiana* and *P. clava*. Captive care protocols developed from this effort will be available as conservation tools

in the future for mitigating loss of a resource due to in-stream activities.

**TAQMAN® MGB REAL-TIME PCR APPROACH TO QUANTIFICATION OF *PERKINSUS MARINUS* AND *PERKINSUS SPP.* IN OYSTERS.** Julie D. Gauthier<sup>1</sup>, Chris R. Miller<sup>2</sup> and Ami E. Wilbur<sup>1</sup>. <sup>1</sup>University of North Carolina, Center for Marine Science, <sup>2</sup>Applied Biosystems.

Several molecular diagnostic assays have been developed in an attempt to replace the traditional Ray's Fluid Thioglycollate Medium (RFTM) assay for detection and quantification of *Perkinsus marinus* in oysters. Real-time PCR technology is now the state-of-the-art method currently used to diagnose disease intensity in vertebrates. We developed a simple (two-reagent) real-time PCR assay to quantify *P. marinus* (PMAR) and *Perkinsus spp.* (PERK) in oysters, using TaqMan® assays designed with Minor Groove Binder (MGB) probes on an Applied Biosystems 7500 Real-Time PCR System. This approach addresses several factors that can interfere with optimal real-time PCR performance. Both PERK and PMAR assays demonstrate strong correlations ( $R^2 \geq 0.99$ ) between parasite cell density and real-time PCR threshold cycle ( $C_T$ ) with amplification efficiencies  $\geq 99\%$ . The PERK assay results in similar amplification plots for the three species tested (*P. marinus*, *P. atlanticus* and *P. andrewsi*), whereas the PMAR assay reported only *P. marinus*. A strong correlation ( $R^2 > 0.90$ ) was found between infection level determined by the traditional RFTM method and quantification by real-time PCR based on internal standards prepared from *P. marinus* spiked oyster tissue. The PCR assays also detected false negatives (15%) diagnosed by the traditional method. We demonstrate the usefulness of these assays in replacing the traditional method for a non-subjective, sensitive, specific and accurate quantification of *Perkinsus spp.* in oyster tissue, with particular application in analyzing frozen or archived material.

**IS HABITAT AVAILABILITY LIMITING RECRUITMENT OF CALICO SCALLOPS (*ARGOPECTEN GIBBUS*)?** Stephen P. Geiger, Janessa C. Cobb and Brett Pittinger. Florida Fish & Wildlife Research Institute, 100 8th Ave. S.E., St. Petersburg, FL 33701.

The Florida calico scallop fishery has declined from a peak of 42.7 million pounds of adductor muscle meats landed in 1984 to none landed in 2005. Removal of scallop and other shell as bycatch may have depleted the essential fisheries habitat for settling veligers. The goal of this study was to describe current abundance and distribution of scallops, shell, and associations between spat and its preferred substrate on two of the historic scallop fishing



grounds. Scallops were collected at 31% of the Atlantic stations, spat at 9%, and both calico scallop shell and other shell at 89%. Spat were most commonly found on scallop shells (46%), but were also found loose, on other shells, rocks and trash. Shell from other molluscs was 1.75 times as abundant as scallop shell. Potential predators that were abundant included *Astropecten articulatus*, *Portunus gibbesii* (also common in the Gulf) and *Distorsio clathrata*. Scallops were collected at 10% of the Gulf of Mexico stations, spat at 8%, scallop shell at 63%, and other mollusc shell at 71%. Rocks and hard bottom were common. Spat preferred other mollusc shells (64%) but were also found loose, on rocks, and on calico scallop shells. Shell from other molluscs was 20 times as abundant as scallop shell. Our findings suggest that the Cape Canaveral calico scallop bed currently has a similar spatial extent to historic records, and that calico scallops are seasonally abundant and are associated with shell deposits. The southwest Florida scallop bed is limited in extent and abundance.

**INGESTION RATES AND LARVAL DEVELOPMENT OF ECHINODERMS ON NATURAL AND ARTIFICIAL PARTICLES.** Sophie B. George, Biology Department, Georgia Southern University, P.O. Box 8042, Statesboro, GA 30460.

Ingestion rates and growth to metamorphosis for five species of echinoderm larvae were investigated in the laboratory using artificial and natural diets. The natural algal diets included *Dunaliella tertiolecta*, *Isochrysis galbana* and *Rhodomonas* sp., and the artificial diet used was a 10-fold diluted artificial feed made from Ziegler E-Z larval diet. The volume of cells ingested varied significantly among species, diets and larval age. All echinoderm larvae ingested significantly higher volumes of the larger alga *Rhodomonas* and the medium-sized alga *D. tertiolecta* than the artificial diet. Younger larvae ingested large and medium-sized particles at lower rates than older larvae. *Dendraster* larvae were more efficient at ingesting both the large and small particles and less efficient in ingesting the medium-sized particles compared to *Strongylocentrotus* larvae. The differences in ingestion rates among species might be due to differences in size, form, water velocity over the ciliated band, density of cilia on the ciliated larval band, and differences in size and palatability of the various diets. Though less of the artificial diet was ingested, *Lytechinus* and *Dendraster* larvae developed to metamorphosis at similar rates and attained similar sizes to those fed *D. tertiolecta* or the mixed algal diet of *I. galbana* and *D. tertiolecta*. An artificial feed would eliminate the need to culture phytoplankton and provide the potential of establishing nutritional requirements for echinoderm larvae. Increasing the size of the artificial particles to 300  $\mu\text{m}$  for younger larvae and 600  $\mu\text{m}$  for older larvae might lead to optimal ingestion rates and the production of large juveniles at metamorphosis.

**THE EFFECTS OF DIETARY CHOLESTEROL AND PHOSPHOLIPIDS ON GROWTH AND PRODUCTION IN THE SEA URCHIN *LYTECHINUS VARIEGATUS*.** Victoria K. Gibbs<sup>1</sup>, Stephen A. Watts<sup>1</sup>, Addison L. Lawrence<sup>2</sup>, Mickie L. Powell<sup>1</sup>, Hugh S. Hammer<sup>1</sup>, Warren T. Jones<sup>1</sup> and John M. Lawrence<sup>3</sup>. <sup>1</sup>University of Alabama at Birmingham, 1530 3rd Ave. South, Campbell Hall, 374, Birmingham, AL 35294-1170. <sup>2</sup>Texas A&M University System, <sup>3</sup>University of South Florida.

Lipids are important components in the diets of marine organisms. In this study, the effects of dietary cholesterol and phospholipids were investigated for small *Lytechinus variegatus* to determine a requirement for either nutrient. *Lytechinus variegatus* ( $n = 16$ ; average initial wet weight =  $13.9 \pm 0.15$  g) were fed semi-purified formulated feeds supplemented to produce low, medium, or high levels of cholesterol (supplemented with 0, 0.3, or 0.6%, as fed) or phospholipids (supplemented at 0, 3, or 4%, as fed) for 12 weeks. Experimental levels of cholesterol and phospholipids were based on established requirements for marine invertebrates. Under the conditions of this study, dietary cholesterol did not have a significant effect on weight gain or gut or gonad production, although total production efficiency was lowest ( $P = 0.007$ ) for individuals fed the low cholesterol feed (36.0, 47.6, and 41.9% for low, medium, and high levels, respectively). Individual weight gain and gonad production were highest for individuals fed the high phospholipid feed (final wet weight = 38.6, 40.3, and 45.8 g,  $P < 0.001$ ; gonad wet weight = 5.3, 6.2, and 6.8 g,  $P < 0.05$ , for low, medium, and high levels, respectively). Total production efficiency was lowest ( $P = 0.001$ ) for individuals fed the low phospholipid feed (35.1, 47.6, and 45.6%, respectively). However, organ production was not substantially affected by cholesterol or phospholipids when adjusted for total individual wet weight (ANCOVA  $P > 0.05$ ). These data suggest that supplemental dietary cholesterol and phospholipids support weight gain in *Lytechinus variegatus*.

**OPTIMIZING METHODS FOR DETECTION OF *CRYPTOSPORIDIUM* OOCYSTS IN NATIVE OYSTERS FROM THE CHESAPEAKE BAY.** Autumn Girouard and Thaddeus Graczyk, Johns Hopkins Bloomberg School of Public Health.

Several groups have documented the presence of *Cryptosporidium*, an anthroponozoonotic enteric parasite, in oysters harvested for commercial purposes. Getting accurate estimates of *Cryptosporidium* contamination levels in oysters is difficult because recovery efficiencies are dependent on the isolation method used. Such estimates are important for determining the human health risks posed by consumption of contaminated oysters in a raw form. In this study, recovery efficiencies are compared for multiple methods used to isolate *Cryptosporidium* oocysts from experimentally contaminated oysters. Oysters were harvested after being allowed to filter oocysts from spiked aquarium water for approximately 24 hours. In one group, the entire oyster meat and all



hemolymph was processed by diethyl ether extraction followed by immunomagnetic separation (IMS) of oocysts from the resulting pellet. In another group, the oysters were dissected so that only gills, digestive diverticula and hemolymph were processed as described above. In the last group, oocysts were isolated directly from hemolymph alone by IMS. Recovered oocysts were enumerated by IFA. Recovery efficiencies for the methods described above were also determined using oyster tissue homogenate and hemolymph spiked with 1000 oocysts, to control for differences in recoveries related to differences in oocyst uptake rates among oysters. The results from this study will be used to determine the best method for quantifying *Cryptosporidium* levels in oysters collected from the Chesapeake Bay. This method would have broad application to native and non-native commercially harvested oysters and would be beneficial for regulatory agencies charged with determining safety of shellfish for human consumption.

**INVESTIGATIONS INTO SOME EARLY-LIFE HISTORY STRATEGIES FOR CARIBBEAN SPINY LOBSTER AND IMPLICATIONS FOR PAN-CARIB CONNECTIVITY.** Jason S. Goldstein<sup>1</sup>, Mark J. Butler IV<sup>2</sup> and Hirokazu Matsuda<sup>3</sup>. <sup>1</sup>University of New Hampshire, <sup>2</sup>Old Dominion University, <sup>3</sup>MPFRC, Japan.

Worldwide, spiny lobsters possess extended larval periods that can easily exceed six months. During this protracted early-life history, pelagic larvae that are produced in one locality disperse throughout oceanic waters, and later settle in far-flung locations to grow and eventually support fisheries in their new home. Observations and limited empirical evidence suggest however that active behavior for Caribbean spiny lobster (*Panulirus argus*) larvae (phyllosoma) and postlarvae (pueruli) appears to have a significant impact on distribution and settlement. In support of this hypothesis, a series of laboratory studies were undertaken to more fully elucidate aspects of phyllosoma and pueruli responses to two selected cues, light and chemical cues, respectively. Phyllosoma culture experiments were initiated to quantify stage duration to the puerulus stage ( $>175 \pm 30$  days after hatch (DAH)) as well as stage-specific differences (vertical movements) in response to simulated oceanic light. Two clear behavioral phototactic shifts to light were observed at ca. 60 and ca. 130 DAH using a custom-made light chamber. A complimentary series of laboratory experiments were carried out to test postlarval orientation and the potential for metamorphic delay in response to chemical cues characteristic of the nearshore nursery environment. Using field-caught animals, results showed that lobster postlarvae are attracted to coastal water as well as to the metabolites of red macroalgae ( $n \approx 300$  trials) in laminar flow chambers. These findings coupled together enhance our understanding of the transport and settlement processes operating for this species throughout the Caribbean and will help to further validate oceanographic lobster models whose management implications are far reaching.

**GENOMICS ON THE HALF-SHELL.** Eric Edsinger Gonzales and Daniel Rokhsar, University of California, Berkeley, Berkeley, CA.

Sequencing of the human genome unveiled Life's working definition of *Homo sapiens*, making it tangible and discrete. Genomic tools and sequencing similarly transformed research in the models systems of development, medicine, and disease and in the process ignited the field of comparative genomics, a self-declared revolution that is now roiling its way across the Tree of Life. In the current third wave of genomics, the focus has been brought to bear on species that are of either phylogenetic or commercial and industrial importance, including model species in alternative energy, agriculture, and aquaculture. Though the revolution might not be televised, aquaculture genomics in bivalves will transform aquaculture and our understanding of its commercially important species, including the oyster *Crassostrea gigas*. As molluscs, bivalves are protostome lophotrochozoans and no fewer than six lophotrochozoan genomes are currently being sequenced. Our own favorite is the gastropod limpet *Lottia gigantea*. Numerous lophotrochozoan and molluscan genomic projects, including ESTs in *C. gigas*, have also been initiated, opening the door to their future genomic sequencing. Here, we review potential application of genomics to the field of aquaculture and how current cellular, biochemical, and ecological techniques can be expanded and integrated with genome-empowered approaches. We then focus on our current research in comparative genomics, looking at developmental patterning of the body plan, shell biomineralization, and metamorphosis in *L. gigantea* and *C. gigas*. Finally, we consider the environmental, economic, and cultural benefits and dangers of genetically modified organisms in aquaculture, a contentious issue that will inevitable be a focus in genomic aquaculture.

**RECOVERY, BIOACCUMULATION AND INACTIVATION OF HUMAN WATERBORNE PATHOGENS BY *CRASSOSTREA ARIAKENSIS*.** Thaddeus K. Graczyk, Autumn S. Girouard, Leena Tamang, Sharon P. Nappier and Kellogg J. Schwab, Johns Hopkins Bloomberg School of Public Health, Baltimore.

Introduction of non-native oysters (i.e., *Crassostrea ariakensis*) into the Chesapeake Bay has been proposed as necessary for restoration of the oyster industry; however, nothing is known about the public health risks related to contamination of these oysters with human pathogens. Commercial size *C. ariakensis* triploids were maintained in large marine tanks with low (8 ppt), medium (12 ppt) and high (20 ppt) salinity water spiked with  $1.0 \times 10^5$  of transmissible stages of the following human pathogens: *Cryptosporidium parvum* oocysts, *Giardia lamblia* cysts and microsporidian spores (i.e., *Encephalitozoon intestinalis*, *Encephalitozoon hellem*, and *Enterocytozoon bieneusi*). Viable oocysts and spores were still detected in oysters on day 33 post water inoculation (pwi), and the cysts on day 14 pwi. The recovery, bioaccumulation, depuration,

and inactivation rates of human waterborne pathogens by *C. ariakensis* triploids were driven by salinity, and were optimal in medium and high salinity water. The concentration of human pathogens from ambient water by *C. ariakensis* and retention of these pathogens without (or with minimal) inactivation and a very slow depuration rate, provides evidence that these oysters may present a public health threat upon entering the human food chain if harvested from polluted water. This conclusion is reinforced by the concentration of waterborne pathogens used in the present study which was representative of levels of infectious agents in surface waters including the Chesapeake Bay. Aquacultures of non-native oysters in the Chesapeake Bay will provide excellent ecological services in regards to efficient cleaning of human infectious agents from the estuarine waters.

**OFFSHORE MOLLUSCAN SHELL ACCUMULATIONS: OCEAN RUBBISH OR REFUGE?** Kaitlin Graiff, Ray Grizzle, Holly Abeels, Jennifer Greene, Melissa Brodeur and Larry Ward. Jackson Estuarine Laboratory, University of New Hampshire.

Molluscan shell accumulations were mapped in a 400 km<sup>2</sup> study area in the western Gulf of Maine off the New Hampshire coast during 2004 using towed underwater video. These shell accumulations were mainly found along the edges of Jeffreys Ledge and were comprised of empty valves of the Atlantic surf clam (*Spisula solidissima*) and the ocean quahog (*Arctica islandica*). Although there has been little ecological research on offshore shell accumulations, they have been identified as potentially significant habitat for several fish species and invertebrates such as lobsters and crabs. Shell accumulations increase the complexity of the sea floor and potentially provide refuge from predators for juvenile life stages of finfish and invertebrates. Subtidal oyster shell (*Crassostrea virginica*) accumulations in southeastern U.S. estuaries are known to provide habitat for many species of commercially important fish and crustaceans. It seems reasonable to expect a similar ecological role for offshore accumulations of shells from other molluscs. Preliminary assessment of the video data from Jeffreys Ledge also indicates a possible relationship between burrowing “anemone forests” (cerianthids) and observed shell accumulations. Future work should explore the habitat value that offshore shell accumulations potentially have for managed species.

**AQUACULTURE CAGE BIOFOULING IN THE GULF OF MAINE: HOW DOES THE BLUE MUSSEL (*MYTILUS EDULIS*) OUT-MUSCLE OTHER SPECIES?** Jennifer Greene and Ray Grizzle, Jackson Estuarine Laboratory, University of New Hampshire.

Biofouling on naturally occurring hard substrates in the Gulf of Maine has been well documented, and a variety of biotic interactions as well as physical factors have been found to influence

settlement and successional patterns. The present study differed from all previous research in the region by focusing on large, offshore fish cages suspended above the seabed. The overall goal was to characterize development of fouling communities, while providing information for engineers and project managers. Biofouling, especially by heavier organisms like molluscs, adds weight and drag to fish cages, thereby reducing water flow and affecting its behavior in rough seas and high currents. Experimental panels were deployed at a depth of ~15 meters over four different time intervals, in replicates of four, over the course of one year. One set of panels was deployed for a full year, two sets for 6-months (beginning at different seasons), and four sets of 3-month (seasonal) panels. Each panel was constructed of a 10 × 10 cm piece of knotless nylon netting attached to a PVC frame. The fouling community that developed diverged from the pattern previously described for shallow water benthos, mainly because of the dominance of the blue mussel, *Mytilus edulis*, in nearly all seral stages as well as the climax (1-year) fouling community. Factors probably influencing this dominance included limited predator abundance, optimal growth conditions, and high reproductive rates.

**DEVELOPMENT OF NOVEL TECHNOLOGIES TO COMBAT FOULING IN AQUACULTURE.** Paul Gribben<sup>1</sup>, Tim Charlton<sup>1</sup>, Lachlan Yee<sup>1</sup>, Rocky De Nys<sup>2</sup> and Peter Steinberg<sup>1</sup>.

<sup>1</sup>Centre for Marine Biofouling, University of New South Wales, Sydney, 2052, Australia, <sup>2</sup>School of Marine Biology and Aquaculture, James Cook University of North Queensland, Townsville, QLD 4811, Australia.

Fouling of nets, pens, and buoys, and in some cases (shellfish) the organisms themselves, are a major problem for the aquaculture industry. In Australia, a conservative estimate of the costs of fouling to the industry is \$20–30 M, in lost production, cleaning and other labor costs, etc. The use of traditional solutions for fouling control (mostly derived from antifouling technologies for ships) is problematic in aquaculture systems because of environmental, health (proximity to food stuffs) and cost constraints. At the centre for Marine Biofouling and Bio-Innovation (University of New South Wales and the School of Marine Biology and Aquaculture (James Cook University)), we have been developing a variety of novel solutions for use in aquaculture. These have developed for a number of industry sectors, and span technologies that are being commercialised to those that are early stage experimental science. Early stage commercialisation technologies are heavy metal free, water based coatings using organic biocides for use on salmon cages and rapid dry coatings for direct application to pearl and edible oysters. New technologies under development include low cost coatings using deterrent surface properties, and living paints encapsulating marine bacteria.

# **IN SITU MEASUREMENTS OF SESTON UPTAKE BY CONSTRUCTED/RESTORED OYSTER REEFS IN SOUTH CAROLINA.**

**R. E. Grizzle<sup>1</sup>, J. K. Greene<sup>2</sup>, L. D. Coen<sup>3</sup> and N. Hadley<sup>3</sup>.** <sup>1</sup>Jackson Laboratory and Department of Zoology, University New Hampshire, 85 Adams Point Rd., Durham, NH 03824. <sup>2</sup>Department of Zoology, University of New Hampshire. <sup>3</sup>MRRI, South Carolina Department of Natural Resources.

One of the metrics that can be used to assess the success of restored oyster reefs is their effect on water quality. This presentation describes a novel technique we have developed to provide rapid assessment of seston uptake (removal) by dense populations of bivalve molluscs, focusing on data gathered during May 2005 from five shellfish reefs of different ages, bivalve densities, and other characteristics in South Carolina. The overall technique involved concurrent sampling upstream and downstream of each study reef with *in situ* fluorometers and pumped water for laboratory analyses. *In situ* fluorometry detected significant differences between mean upstream and downstream readings for all five reefs, with total seston uptake ranging from 1.9% to 23.1%. Laboratory analysis of chlorophyll *a* concentrations in pumped water samples taken concurrently with the *in situ* fluorometry data showed substantial variability between upstream and downstream data, except for one reef which indicated an overall 35% uptake rate (compared to 23.1% based on *in situ* fluorometry data). As a control, sampling over recently constructed reefs (shell only) did not result in any measurable seston loss from the water column. Our system is a fast and effective approach to quantifying seston removal, and when data are logged at short-term intervals (seconds) potentially provides information on variations in feeding rates and related processes typically only available in laboratory studies.

# **IMPACTS OF INVASIVE SPECIES ON NATIVE OYSTER RESTORATION IN CENTRAL CALIFORNIA.**

**Edwin Grosholz<sup>1</sup> and David Kimbro<sup>2</sup>.** <sup>1</sup>Department of Environmental Science and Policy, <sup>2</sup>Bodega Marine Laboratory, University of California, Davis, CA 95616.

The invasion of non-native species in the coastal waters of the U.S. has had enormous impacts on community structure and ecosystem function. Invasive non-native species have been particularly problematic where attempts have been made to restore native species. We document the impacts of invasive predatory crabs and oyster drills on attempts to restore native Olympia oysters (*Ostreola conchaphila*) in the Tomales Bay estuary in central California. Our work shows that introduced European green crabs (*Carcinus maenas*) can have a significant predatory impact on outplanted juvenile oysters in field enclosure experiments and in experimental mesocosms. We also demonstrate that that introduced oyster drills (*Urosalpinx cinerea*) are also significant predators in both field experiments and mesocosm experiments. The distribution of these predators shows a strong density gradient with

the estuarine conditions in Tomales Bay and parallels an equally strongly gradient in recruitment, survival and growth of native oysters. We discuss the degree to which these introduced predators shape the population structure of native oysters and how they will influence future efforts to restore oysters in central California.

# **EARLY STEAMER CLAM (*MYA ARENARIA*) IN TWO COASTAL PONDS IN OAK BLUFFS, MA (MARTHA'S VINEYARD ISLAND).**

**David W. Grunden and Danielle Ewart,** Shellfish Department, Town of Oak Bluffs, P.O. Box 1327, Oak Bluffs, MA, 02557.

The investigation into this project began in 2001 when the Town of Oak Bluffs purchased 200,000 seed (2–3 mm) steamer clams. Three trial restoration sites were chosen; two in Sengekontacket Pond and one in Lagoon Pond. One site in Sengekontacket Pond has remained open for harvest. The other two sites are alternated one opening every other year. All three sites are utilized primarily for recreational shellfishing, though occasionally a commercial shellfisherman does harvest from one of the sites in Sengekontacket Pond. The first year the steamer seed was only grown in the upweller and broadcast seeded at 12–15 mm. The seed at this size dug into the sandy substrate within 15 minutes. The area was marked but ice removed the marker that first winter. In subsequent years the Town of Oak Bluffs has been able to increase the amount of seed raised to 600,000 in 2005 (another 600,000 has been ordered for the 2006 season). The Town's Shellfish Department constructed sand filled nursery rafts to allow for greater growout of the seed. The seed steamers are still removed from the upweller once they reach 12–15mm, but are now transferred into the nursery rafts where they are held until late mid-September or early October. The seed measured 22.4–38.2 mm when they were planted out in the fall of 2005. The fishing effort has been monitored by the Shellfish Department and we have seen a modest increase in the steamer clam landings from these areas since the project began.

# **GENOME MAPPING IN THE EASTERN OYSTER (*CRASSOSTREA VIRGINICA* GMELIN).**

**Ximing Guo, Yongping Wang, Ziniu Yu, Lingling Wang and Jeong-ho Lee.** Haskin Shellfish Research Laboratory, Rutgers, The State University of New Jersey, 6959 Miller Ave., Port Norris, NJ 08349.

The eastern oyster, *Crassostrea virginica* Gmelin, is an important aquaculture species as well as a popular model species for molluscan research. The biology and ecology of the eastern oyster are well understood, but our knowledge about its genome remains limited. We have been developing tools and applying them in genome mapping in the eastern oyster. For physical mapping, we used fluorescence *in situ* hybridization (FISH) to characterize and map oyster chromosomes. Repetitive DNA sequences and genes were mapped to oyster chromosomes by FISH, and some revealed interesting features of the oyster genome. The chromosomal loca-

tion of rRNA genes, on the long arms of Chromosome 10 in Pacific species and on the short arms of Chromosome 2 in Atlantic species, provided an interesting divide between the Pacific and Atlantic species. Nine PI clones were mapped to seven of the ten oyster chromosomes. For linkage mapping, we developed over 600 amplified fragment length polymorphism (AFLP) markers and constructed four moderately dense linkage maps. We identified disease-resistance QTL by mapping markers that showed significant frequency shifts after disease-inflicted mortalities. Markers affected by diseases clustered together on genetic maps, and all affected markers within a cluster showed frequency shifts in the same direction. Mapping analysis in two families identified 12 putative Dermo/summer mortality-resistance QTL, and at least seven were independent. Microsatellites are being developed and mapped to increase the transferability of genetic maps and mapped QTL. Candidate host-defense genes are being mapped using single nucleotide polymorphism to detect possible linkage to disease-resistance QTL.

**OYSTERS AND OYSTER FARMING IN CHINA: A REVIEW.** Ximing Guo<sup>1</sup>, Guofan Zhang<sup>2</sup>, Lumin Qian<sup>3</sup>, Haiyan Wang<sup>1</sup>, Xiao Liu<sup>2</sup> and Aimin Wang<sup>4</sup>. <sup>1</sup>Haskin Shellfish Research Laboratory, Rutgers, The State University of New Jersey, 6959 Miller Ave., Port Norris, NJ 80349. <sup>2</sup>Institute of Oceanology, Chinese Academy of Sciences, PRC. <sup>3</sup>Third Institute of Oceanology, Oceanic Administration, PRC. <sup>4</sup>Ocean College, Hainan University, PRC.

In an effort to survey *Crassostrea ariakensis* populations in China, we conducted literature reviews and visited over 37 sites along China's coast. Here we present our findings about oysters and oyster farming in China in light of recent taxonomic revisions. Seventeen species of oysters have been reported along China's coast. Many of the species occur in southern China and are relatively rare. There is considerable confusion about the classification of *C. ariakensis* and three other species. Numerous oyster reefs, both ancient and living, exist along the coast, and *C. ariakensis* was present in all four live reefs that we saw. In three of the reefs, *C. ariakensis* is the dominant or founding species, where large oysters on the bottom are *C. ariakensis* and small oysters on top are other species. Oyster farming is primarily for *C. hongkongensis* in the south (Guangxi and Guangdong), *C. gigas* in the north (Shandong and Liaoning), and *C. angulata* in the middle (Fujian and Zhejiang). *C. hongkongensis*, also known as the white oyster or *C. rivularis*, is one of the most important species cultured in China. *C. hongkongensis* culture is based on natural seeds with one estuary in Guangxi supplying about 2.5 billion. Most of the published literature on *C. rivularis* from southern China is for *C. hongkongensis*, not for *C. ariakensis*, which is not intentionally cultured. It is present at low frequencies in natural *C. hongkon-*

*gensis* seeds and selected against by farmers. Pollution has devastated oyster populations in at least two estuaries.

**TESTING DISEASE RESISTANCE IN OYSTERS: EXPERIMENTAL INFECTIONS WITH BACTERIAL PATHOGENS IN *CRASSOSTREA VIRGINICA* LARVAE AND SPAT.** Javier Gómez-León<sup>1</sup>, Dale Leavitt<sup>2</sup>, Karen Tammi<sup>2</sup>, Rachel Hadley<sup>1</sup>, Luisa Villamil<sup>1</sup> and Marta Gómez-Chiarri<sup>1</sup>. <sup>1</sup>University of Rhode Island, Department of Fisheries, Animal and Veterinary Science, 20A Woodward Hall, Kingston, RI 02881. <sup>2</sup>Roger Williams University.

Culture of the eastern oyster (*Crassostrea virginica*) is a traditional activity that has great economical importance in the East Coast of USA and the Gulf of Mexico. Globally, shellfish production is often affected by bacterial pathogens, mainly *Vibrios*, which lead to high mortality rates in shellfish hatcheries. Another bacterial disease that has heavily impacted oyster culture in the Northeast US is Juvenile Oyster Disease (JOD), thought to be caused by *Roseovarius crassostreae*. In the present study, bacterial isolates that caused important mortalities in Pacific oyster larvae, RE22 and RE101, as well as an isolate from JOD-affected oysters (CV919-312) were used to perform experimental infections of oyster larvae and spat in order to determine differences in the susceptibility to bacterial infection of three oyster lines: a local Rhode Island line, a line resistant to Dermo and MSX (NEH), and a line resistant to JOD (FMF). All bacterial isolates tested were able to induce significant mortality in larvae and spat of *C. virginica*, reaching mortalities ranging from 50–100%. Differences in susceptibility between the lines were observed, with the NEH line showing the highest survival. Infected larvae exhibited abnormal circular swimming movements on their sides and deformed velum with cilia clumping. Factors affecting survival included temperature and size of the oysters. This research may provide a useful tool to test for disease resistance mechanisms in oysters.

**EFFECT OF DIETARY PROTEIN ON CONSUMPTION, GROWTH AND PRODUCTION OF THE SEA URCHIN *LYTECHINUS VARIEGATUS*.** H. Hammer<sup>1</sup>, S. Watts<sup>1</sup>, A. Lawrence<sup>2</sup> and J. Lawrence<sup>3</sup>. <sup>1</sup>Department of Biology, University of Alabama at Birmingham, <sup>2</sup>Shrimp Mariculture Project, Texas A&M University, <sup>3</sup>Department of Biology, University of South Florida.

Feeds that differ in protein concentration (17, 21, 25 and 31%, as fed) were formulated, cold-extruded and dried at 60°C to produce a dry pellet. Sea urchins ( $n = 64$ ) were divided into four groups and placed individually into a semi-recirculating system at  $32 \pm 2\%$  salinity and  $22 \pm 1^\circ\text{C}$ . Urchins were fed daily one of four diets *ad libitum* for 87 days. Consumption was measured daily; urchins were weighed and test diameters measured at days 0, 29,

57 and 87. At day 87, urchins were dissected and separated into components (test, lantern, gut and gonad) that were weighed, dried and reweighed. At days 0–87, urchins fed the 31% protein feed consumed less food than the other treatments. Urchins fed the 31% protein feed had a significantly greater body weight gain/day (1.3%) and test diameter (42.7 mm) than urchins fed the 17% (0.9%, 38.3 mm, respectively) or 21% protein feed (1.1%, 40.7 mm, respectively) but not the 25% protein feed (1.3%, 41.7 mm, respectively). Wet weight was significantly different among the treatments and varied directly with dietary protein (40.4, 37.0, 34.0, 28.6 g, respectively). Urchins fed the 31% protein feed had the highest production efficiency (48.1%) among the treatments (36.6, 32.5 and 24.8%, respectively). Gonad production efficiency in urchins fed the 31% protein feed (12.6%) was greater than urchins fed the 17% (6.9%) or 21% (9.4%) but not the 25% protein feed (10.7%). The 31% protein feed provided the best weight gain with the highest efficiency of the feeds tested.

**DEMOGRAPHIC CHARACTERISTICS OF STABLE FRESHWATER MUSSEL POPULATIONS IN THE SOUTH-EASTERN UNITED STATES.** Wendell R. Haag and Melvin L. Warren Jr., USDA Forest Service Center for Bottomland Hardwoods Research, 1000 Front St., Oxford, MS 38655.

Population dynamics of freshwater mussels are poorly known and represent a major information need for conservation of these imperiled animals. Most notably, the level of recruitment needed to maintain a stable mussel population is unknown, making it difficult to meaningfully assess the viability of a population. We measured annual recruitment and survivorship in two diverse mussel communities (Little Tallahatchie River, MS, and Sipsey River, AL) from 1999 to 2005. For all species, survivorship from glochidia to the recruit stage (benthic individuals approximately two to four months of age) was low ( $9.12 \times 10^{-6}$  to  $3.92 \times 10^{-5}$ ), but was high for all other life stages (0.63–0.95). Recruitment varied widely among species, sites, and years. For some species (e.g., *Amblema plicata*, *Elliptio arca*, and *Obovaria unicolor*), recruitment was sporadic, ranging from years with no detectable recruitment to years in which recruits composed >50% of the population. For other species (e.g., *Fusconaia cerina*, *Pleurobema decisum*, *Quadrula asperata*, and *Q. pustulosa*), recruitment occurred at a more constant level (0–15% of population). We used stochastic stage-based matrix population models based on observed demographic parameters to evaluate the potential influence of varying levels of recruitment on long-term population viability. These models predict that species with higher annual variability in recruitment require a higher mean recruitment level over time to maintain a stable population than species with less variable recruitment. Demographic differences among species show the existence of widely divergent life history strategies in freshwater

mussels and have important implications for the conservation of these animals.

**IMPACT OF SEA OTTERS ON SHELLFISH FISHERIES AND AQUACULTURE IN B.C. CANADA.** Rick Harbo, Linda Nichol, Laurie Convey, Jennifer Toole and Lieneke Marshall. Fisheries and Oceans Canada, 3225 Stephenson Point Rd., Nanaimo, B.C., V9T 1K3, Canada.

Re-introduced sea otters have increased in number (>3,200 estimated in 2005) and expanded their range on the British Columbia coast, causing impacts on shellfish fishery resources. There have been concerns expressed by First Nations about the loss of access to clam, crab and sea urchin resources. The increase in kelp beds (a result of otter predation on urchins) has affected local navigation channels for small boats. A variety of commercial and recreational shellfish fisheries have been impacted by sea otters, including Dungeness crab, *Cancer magister*, red sea urchin, *Strongylocentrotus franciscanus*, Geoduck clam, *Panopea abrupta*, and Manila clam, *Venerupis philippinarum*. While otters are not the only cause of shellfish declines off B.C. (e.g., northern abalone, *Haliotis kamtschatkana*), predation by otters will keep shellfish abundances and sizes at levels that hinder the resumption of commercial fisheries. Sea otters have limited plans for geoduck aquaculture and enhancement on the west coast of Vancouver Island. There have been complaints about otter predation on some intertidal manila clam aquaculture tenures. To date, sea otters have taken little interest in Pacific oysters on intertidal or deep water aquaculture tenures. It is expected that conflicts will continue and increase in the next 10 years.

**AGE AND GROWTH OF WILD CRASSOSTREA ARIAKENSIS AND C. GIGAS FROM LAIZHOU BAY, CHINA.** Juliana M. Harding and Roger Mann. VIMS, P.O. Box 1346, Gloucester Point, Virginia, USA 23062.

Shell height at age estimates from Suminoe (*Crassostrea ariakensis*) and Pacific (*Crassostrea gigas*) oysters from a natural oyster reef in Laizhou Bay, China were compared with estimates from triploid *C. ariakensis* of known age from the Rappahannock River, Virginia. Both *C. ariakensis* and *C. gigas* reach shell heights in excess of 76 mm (3 inches) within two years after settlement regardless of the source location. This fast growth appears to continue through at least age four or age five in wild individuals as the growth trajectory for both species had not begun to flatten in the oldest individuals collected. Fitted growth curves were not significantly different between species within the same habitat, within species in different habitats or between species in different habitats.

**TROPHIC CONSEQUENCES OF A LONG-LIVED NONNATIVE PREDATOR (*RAPANA VENOSA*) ON ESTUARINE COMMUNITY DYNAMICS.** Juliana M. Harding and Roger Mann. VIMS, P.O. Box 1346, Gloucester Point, Virginia, USA 23062.

Veined rapa whelks, *Rapana venosa*, are large generalist predators with the potential to live in excess of 10 yrs and life history suitable for successful invasion of estuarine habitats. The presence of rapa whelks in a habitat has obvious consequences for the prey field in that rapa whelk prey consumption shifts ontogenetically from small (e.g., *Mytilus* sp., *Macoma* sp., *Mya* sp., *Crassostrea* sp.) to large (e.g., *Mercenaria* sp., *Crassostrea* sp.) prey. Laboratory and mesocosm experiments indicate that wild rapa whelks reach a size refuge from predation by blue crabs (*Callinectes sapidus*) within one year post settlement at critical sizes of 30–40 mm SL. At sizes >40 mm, rapa whelk distribution in Chesapeake Bay is probably limited only by salinity and food availability. The presence of large predators with the ability to repel competitors (blue crabs) on shared feeding grounds may force native species into habitat refugia delimited by salinity tolerances. Scenarios predicting relative abundance and competitive interactions between bivalve prey, rapa whelks, and blue crabs with impacts on trophic dynamics and habitat use are discussed.

**GENETIC TRACKING OF RESTORATION OYSTERS TO GAUGE SUCCESS—A COST/BENEFIT ANALYSIS.** Matthew P. Hare, University of Maryland.

Restoration of oyster stocks in Chesapeake Bay is a monumental task to which abundant resources have been committed and for which many dedicated parties have labored. Beyond establishing a ten-year goal for the increase of census numbers, little attention was initially paid to establishing rigorous benchmarks for success, or collecting data that could either measure small successes or determine the cause of failures. This has been changing more recently, but now most restoration effort is focused on targeted population supplementation using artificially selected, disease tolerant *C. virginica* to combat high mortalities from parasitic diseases. This tactic has potential benefits and considerable risks. I will argue that genetic testing of recruitment is necessary to evaluate and manage the genetic risks of supplementation with inbred oysters, but it also provides the most meaningful measure of overall restoration efficacy. Results will be presented from a highly collaborative effort to genetically monitor DEBY-strain restoration plantings in two Chesapeake subestuaries. The results indicate that the hatchery amplification of DEBY broodstock to produce restoration oysters is contributing to inbreeding in the oysters over and above that already realized during artificial selection. Also, the DEBY contribution to local recruitment in the Great Wicomico River in 2002 was approximately 10%, a result that belies appearances based on the number of DEBY oysters planted and the

elevated levels of recruitment that year. Inbreeding may or may not be the greatest risk facing restoration oysters and threatening restoration success, but only improved genetic testing will allow informed risk management.

**METABOLIC RATES OF *CRASSOSTREA ARIAKENSIS* AND *CRASSOSTREA VIRGINICA* AT TWO TEMPERATURES AND THREE SALINITIES.** Nicole Harlan, Kennedy Paynter and Donald Meritt. University of Maryland Center for Environmental Science.

Maryland and Virginia have proposed to replace the native oyster, *Crassostrea virginica*, with the suminoe oyster, *Crassostrea ariakensis*, in Chesapeake Bay. *C. virginica*, is highly tolerant of hypoxic conditions and can survive emersion or nearly anoxic seawater for days to weeks depending on the temperature. In order to replace *C. virginica*'s ecological niche of establishing vast benthic reefs in Chesapeake Bay, *C. ariakensis* may require similar tolerances. However, when the oysters were placed in sealed jars of anoxic water, *C. ariakensis* lived for an average of four days, while *C. virginica* persisted for more than fourteen days. Studies at 22°C have shown that the metabolic rate of *C. ariakensis* ( $1.96 \pm 0.102$  O<sub>2</sub>/hr/gdw) is significantly higher than that of *C. virginica* ( $1.15 \pm 0.079$  mg O<sub>2</sub>/hr/gdw;  $p = 0.0244$ ). In order to better understand the aerobic requirements of these two species under different conditions, standard metabolic rates of each species were determined at two temperatures, 10 and 20 °C, and three salinities, 5, 15, and 25 psu. At both temperatures and all three salinities, the metabolic rate of *C. ariakensis* was higher than that of *C. virginica*. Upon immersion in the test chambers, *C. ariakensis* gaped and began using oxygen within minutes, while *C. virginica* kept their valves shut for much longer. These data may influence the decision to use *C. ariakensis* as an ecological substitute for *C. virginica* in the Chesapeake Bay.

**ANALYSIS OF ENZYME, CARBOHYDRATE AND MINERAL DISTRIBUTION IN THE FOOT OF ABALONE SHELLFISH.** Leanne Harris, Helen Lambkin and Nuala O'Byrne-ring. Dublin Institute of Technology, Biological Sciences, Kevin Street, Dublin 8, Ireland.

The meat of the abalone shellfish has been labelled as a luxury food for thousands of years. The source of this epicurean delicacy is the foot, which is the most conspicuous external feature of this organism. The foot is a large muscular organ with an extensive nerve and vascular supply that serves both sensory and locomotory functions. The foot is also involved in many other functions such as locating and manipulating food, attaching eggs to substrates, cleaning the shell, finding potential mates and thwarting predators.

The foot is primarily made up of epithelial tissue, connective tissue and muscle. In this study the distribution of functional and structural elements was investigated in the pedal organ of two species of abalone, *Haliotis tuberculata* and *Haliotis discus hannai*. The pedal and peripheral epithelia expressed high levels of activity for the following enzymes: chloroacetate esterase,  $\alpha$ -naphthyl butyrate esterase, alkaline and acid phosphatase, peroxidase and carbonic anhydrase. The sub-epithelial ganglion cells were positive for  $\alpha$ -naphthyl butyrate esterase and acetylcholinesterase. Neutral mucins, acid mucins, carboxylated mucins and sulphated mucins were found in epithelial cells, in sub-epidermal gland cells and in the ground substance of the connective tissue and muscle. Melanin was identified in the sub-epidermal gland cells and in the pedal and peripheral epithelial cells. The basement membrane of the pedal epithelium was positive for calcium. A myriad of cell components and cellular activities in the tissues of the abalone foot were demonstrated, revealing cell types and reflecting the molecular pathways at work within these tissues.

**DEVELOPMENT OF PCR TECHNIQUES FOR THE DETECTION OF *VIBRIO CARCHARIAE* AND *PERKINSUS OLSENI* IN ABALONE TISSUES.** Leanne Harris, Fergus Ryan, Helen Lambkin and Nuala O'Byrnering, Dublin Institute of Technology, Biological Sciences, Kevin Street, Dublin 8, Ireland.

Global demand for abalone has significantly increased in recent years, however, wild stocks of abalone have declined in number, as a result of overexploitation and the spread of infectious disease. Disease outbreaks are recognised as a significant constraint to aquaculture production and trade that affects both economic development and the socio-economic revenue of many countries. Bacteria and protozoa are the most commonly encountered pathogens in abalone shellfish. *Vibrio harveyi/carchariae* is a highly pathogenic bacterium to abalone and *Perkinsus atlanticus/olseni* is a protozoan that also causes severe disease in this shellfish. Both pathogens are ranked amongst the top ten most significant disease causing organisms of abalone. In this study, a multiplex PCR method was developed to simultaneously amplify a 413 bp region of the 16S rRNA sequence of *V. carchariae/harveyi* and a 155 bp region of the actin mRNA gene sequence of *Haliotis* spp. This multiplex PCR was used to amplify these sequences in both fixed tissues and paraffin embedded tissues of infected *Haliotis tuberculata*. A primer set was designed to target a 245 bp region of the ITS sequence of *P. atlanticus* from paraffin embedded samples of infected *Ruditapes decussatus* which could be adapted to detect *P. olseni* in abalone tissues. Also quantitative PCR using these primers is a further potential development. These PCR protocols offer a rapid and specific method for the identification of *V. carchariae* and *P. olseni* in shellfish.

**COMPARATIVE GROWTH AND SURVIVAL OF DIPLOID AND TRIPLOID SUMINOE OYSTERS, *CRASSOSTREA ARIAKENSIS*, IN MULTIPLE QUARANTINE SYSTEMS.** Heather D. Harwell and Standish K. Allen Jr, Virginia Institute of Marine Science, P.O. Box 1346, Gloucester Point, VA 23062.

Much of the research on *Crassostrea ariakensis* has revealed superior growth rates and resistance to disease compared to the native oyster, *C. virginica*. All field studies of growth and survival have utilized sterile triploid oysters for reasons of biosecurity. Thus, triploids are serving as a surrogate for diploid performance in these field trials. A direct, simultaneous comparison of the growth and survival of diploid and triploid *C. ariakensis* is needed to refine population growth models based on triploid field data. Three replicate lines of diploid and triploid *C. ariakensis* were placed at four quarantine systems in Virginia and Maryland in December 2004. Individual repeated measures of subsets of oysters were gathered from monthly data on percent survival, wet weight, and shell length. In addition, quarterly estimates of condition index were obtained. Data gathered in this fashion will be used to determine a correction factor(s) that can then be applied to results from past studies of triploid *C. ariakensis* in order to refine models of potential population growth.

**ROLE OF LOW SALINITY REFUGE IN REGULATING THE PREVALENCE OF THE PARASITE *LOXOTHYLACUS PANOPAEI* IN THE XANTHID *PANOPEUS OBESUS*.** Lesli Haynes<sup>1</sup>, S. Gregory Tolley<sup>1</sup>, Aswani K. Volety<sup>1</sup> and James T. Winstead<sup>2</sup>, <sup>1</sup>Florida Gulf Coast University, 10501 FGCU Blvd. S., Ft. Myers, FL 33965, <sup>2</sup>United States Environmental Protection Agency.

This study was conducted to examine the potential influence of salinity, a proxy for freshwater inflow, on the prevalence of the castrator parasite, *Loxothylacus panopaei*, on mud crabs found on Southwest Florida oyster reefs. Spatial and seasonal patterns of the presence of potential host crabs and the prevalence of the parasite were assessed in the Caloosahatchee, Estero, and Faka-Union estuaries of Southwest Florida. Lift nets (1 m<sup>2</sup>) containing five liters of oyster clusters were deployed on intertidal reefs at three sites along the salinity gradient of each estuary. Nets were deployed during three seasonally dry and three seasonally wet months for a period of 30 d. Although *Panopeus obesus* were collected at all three locations within each estuary, densities tended to increase downstream in higher salinity waters. Parasite prevalence decreased at the upper stations in each estuary, was reduced during wet months compared to dry months, and was lower for those estuaries that experienced high levels of freshwater inflow. Furthermore, parasite prevalence was positively correlated with the mean salinity at capture of host crabs. Based on the distribution of *P. obesus* and the above patterns related to salinity, it appears that freshwater inflow and seasonal rains might regulate the prevalence



of this castrator parasite in Southwest Florida by creating spatiotemporal, low salinity refuge for its host.

#### MAPPING GENES AFFECTING SHELL COLOR AND SHAPE IN THE PACIFIC OYSTER *CRASSOSTREA GIGAS*.

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Variation in the color and shape of oyster shells is widely believed to be environmentally determined. However, Brake et al (2004 Aquaculture 229:89) recently provided evidence that shell and mantle edge color in the Pacific oyster, *Crassostrea gigas*, are heritable. We observed variation in shells of an F<sub>2</sub> family of Pacific oysters that appeared to be inherited, and we tested this hypothesis by statistical analyses and genetic mapping. Measures of shell shape, size and color were made from digital photographs of 179 oysters, using ImageJ software. Principal component analyses were used to consolidate the measured traits, and mapping was done on both principal components and some of the original individual traits. Mapping was made possible by a previously compiled linkage map for this same family of oysters, comprising 59 microsatellite DNA markers on 11 linkage groups. Using an interval mapping method, we identified a quantitative trait loci (QTL) for pigment saturation of the left valve on linkage group VIII, between *ucdCgi183* and *ucdCgi184*; genotypes at *ucdCgi183* explain 32% of shell color variance. Similarly, we identified two QTL for a pronounced left bend in some anteriorly viewed valves ("hook hinge") on linkage group III, in the adjacent intervals of *cmrCgi-ucdCgi198-imbCgi49*; genotypes at *ucdCgi198* explain 32% of hook hinge variance. Left hook hinge appears to be recessive, while shell pigmentation is nearly additive. These observations suggest that genes, as well as environment play, a role in determining the color and shape of oyster shells. Markers associated with these QTL could be used to breed oysters with more desirable shell characteristics.

#### BIVALVE SHELLFISH CAN BE VECTORS OF TRANSPORT OF HARMFUL ALGAE. Hélène Hégaret<sup>1</sup>, Sandra E. Shumway<sup>1</sup> and Gary H. Wikfors<sup>2</sup>.

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Commercially-exploited bivalve molluscs are moved from one body of water to another for different purposes. Our study is testing the hypothesis that harmful algae can be introduced into new environments by means of these shellfish relocations. We identified which managed shellfish species and HABs co-occur geographically and established an experimental protocol to screen

shellfish-HAB pairs for consumption of the algae and release of living propagules after removal from the HAB exposure. Several cultured strains of harmful algae, such as *Prorocentrum minimum*, *Alexandrium fundyense*, and *Heterosigma akashiwo*, were fed to various species of bivalve molluscs, *Crassostrea virginica*, *Argopecten irradians*, *Mercenaria mercenaria* and *Mytilus edulis*, to assess the ability of the algal cells to pass intact through the digestive tract and subsequently grow. Ten bivalves of each species were also exposed for two days to a simulated harmful algal bloom at a natural bloom concentration. The algae were removed after two days of exposure, and the bivalves were kept for two more days in ultrafiltered seawater. Biodeposits were collected and observed under the microscope after 24 and 48 additional hours to evaluate the presence or absence of intact, viable cells or temporary cysts of the algae. Subsamples of biodeposits were transferred into algal culture medium and filtered seawater and monitored microscopically for algal growth. Intact algal cells of *P. minimum*, *A. fundyense*, and *H. akashiwo* were seen in biodeposits; generally these re-established growing populations.

#### EFFECT OF *IN VITRO* INTERACTIONS BETWEEN *MERCENARIA MERCENARIA* HEMOCYTES AND SEVERAL SPECIES OF HARMFUL ALGAE. Hélène Hégaret<sup>1</sup>, Gary H. Wikfors<sup>2</sup>, Madeleine Gonçalves<sup>3</sup> and Sandra E. Shumway<sup>1</sup>.

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Shellfish species are very often exposed to harmful algal blooms (HABs), which can have noxious effects. The northern quahog (= hard clam), *Mercenaria mercenaria*, can experience blooms of several HAB species, such as *Prorocentrum minimum*, *Heterosigma akashiwo* or *Alexandrium fundyense*. *In vitro* tests of interactions between those harmful algae and *M. mercenaria* hemocytes have been conducted, measuring possible differences in hemocyte parameters attributable to harmful algae and measuring also the effect of hemocytes on the algae themselves. Indeed, the purpose of these experiments was to understand the possible roles of the hemocytes in bivalve responses to HABs, and how the algal cells are affected by these responses. Using microscopic and flow-cytometric observations, changes in morphology and physiology of the hemocytes, and of the algal cells, were assessed. In hemocytes, changes in hematology and physiology, including cell concentration, mortality, phagocytosis, adhesion, apoptosis and oxidative burst response, were determined. Changes in the physiology and the characteristics of the algal cells were determined as well: mortality, size, chlorophyll fluorescence, and internal complexity. The results show differences in the hemocyte responses when exposed to the several harmful algae tested. Examples of hemocyte responses include phagocytosis of algal cells or adhesion around cells, with attendant increases in dead, achlorotic algal cells. Thus, *in vitro* tests allow a better understanding of the role of



the hemocytes and the hemolymph in the defense mechanisms in molluscan shellfish to harmful algal cells.

**DEVELOPMENT AND APPLICATION OF MICROSATELLITE MARKERS TO DETERMINE THE IMPACT OF RESTORATION OF THE BAY SCALLOP.** Elizabeth Hemond and Ami E. Wilbur, University of North Carolina Wilmington, Department of Biology and Marine Biology, 601 S. College Rd., Wilmington, NC 28403.

A number of marine species, including the bay scallop (*Argopecten irradians*), are being restored using aquaculture-propagated broodstocks. Because these projects are laborious and expensive, it is important to be able to quantify the impact of these restorations. Natural genetic variation within species can be used to distinguish between the progeny of native populations and restoration stocks and thereby quantify the relative impact, provided sufficient genetic markers are available, and that the native and restoration stocks are differentiated to some degree. To facilitate these efforts, we have developed a set of microsatellite loci for bay scallops. Microsatellites are highly polymorphic, tandemly repeated sequences of two to six nucleotides dispersed throughout the genome of most eukaryotic organisms. Due to their number and variability, microsatellites are a powerful tool for identifying differences among populations (even individuals) and when used in conjunction with multi-locus analytical methods can provide the requisite discrimination needed to quantify the impact of restoration. We have isolated 19 trinucleotide and 41 tetranucleotide microsatellite loci and are in the process of characterizing the markers with respect to the level of polymorphism exhibited, presence of null alleles, and fidelity of inheritance. Preliminary data on four loci (AICL112, AICL115, AICL131, and AICL271) revealed extensive polymorphism (37, 14, 17 and 10 alleles, respectively) in two populations from Florida (Anclote Estuary and Pine Island Sound), but surprisingly little differentiation between locations (Monte Carlo  $\chi^2$ , allele frequency distribution,  $p > 0.05$ ). Results from additional loci will be presented and the utility of these markers in assessing restoration will be discussed.

**MANGROVE OYSTERS AS BIOINDICATORS IN SUB-TROPICAL DRY CLIMATES.** Heidi Hertler<sup>1</sup>, Lindsay Martinez<sup>1</sup>, Danielle Kreeger<sup>2</sup> and Graciela Ramirez-Toro<sup>1</sup>. <sup>1</sup>Inter-American University of Puerto Rico, <sup>2</sup>Partnership for the Delaware Estuary.

Marine systems of the Caribbean are increasingly impacted by changes in land use. Escalating development is in many places out-pacing the ability of existing upland, salt flats, and mangroves to intercept sediment and nutrient runoff flowing toward the adjacent marine system. Elevated concentrations of chlorophyll-*a* and total suspended solids were correlated with land development in Southwest Puerto Rico. However, a direct cause and effect

relationship has not yet been established between development on the land and water quality degradation in the adjacent marine community. Suspension-feeding bivalves are regarded as excellent bioindicators of water quality, but they have not been studied in this context in tropical waters. We examined the population size structure and physiological condition of mangrove oysters, *Crassostrea rhizophorea*, along a gradient of land development in Southwest Puerto Rico in a first step towards using this species as a bioindicator of water quality in sub-tropical environments. Data suggest that *C. rhizophorea* fares better in undisturbed, clearer water rather than in eutrophied areas near development where food availability may be greater. In addition, oyster health, as measured by condition index, was inversely correlated with increasing land use. Thus, physiological measures of *C. rhizophorea* fitness may be useful indicators of ecosystem integrity.

**CAN HARD CLAM LARVAL SURVIVAL EXPLAIN RECRUITMENT FAILURE IN GREAT SOUTH BAY: A MODELING STUDY?** Eileen E. Hofmann<sup>1</sup>, Eric N. Powell<sup>2</sup>, John M. Klinck<sup>1</sup>, John N. Kraenter<sup>2</sup>, Rebecca Marzec<sup>2</sup> and V. Monica Bricelj<sup>3</sup>. <sup>1</sup>Old Dominion University, <sup>2</sup>Haskin Shellfish Research Laboratory, Rutgers University, <sup>3</sup>Canada Natural Resources Council.

A biochemically-based model was developed to simulate the growth, development, and metamorphosis of hard clam (*Merccenaria mercenaria*) larvae. Larvae are simulated in terms of protein, neutral lipid, polar lipid, carbohydrate, and ash content. Initial biochemical content of the larva is determined by egg composition and changes in this occur as the larva grows and in response to the biochemical composition of available food. Simulations that used environmental conditions from Great South Bay, Long Island showed that variations in temperature and food quantity produce small changes in overall larval survivorship relative to that obtained from average conditions. The largest decrease in larval survivorship resulted from variations in the quality of food available to the larva. Reductions in food lipid content resulted in lowest larval survival rate. These results suggest that changes in food quality during the past decade may be a contributing factor to the present low recruitment rates of hard clam larvae in Great South Bay.

**LINKING MARINE PATHOGENS TO MOLLUSCAN SHELLFISH: THE ECOLOGICAL ROLE OF MARINE AGGREGATES.** Bridget A. Holohan<sup>1</sup>, M. Maille Lyons<sup>1</sup>, J. Evan Ward<sup>1</sup>, Roxanna M. Smolowitz<sup>2</sup>, Kevin R. Uhlinger<sup>2</sup>, Joseph J. Vallino<sup>2</sup> and Bassem Allam<sup>3</sup>. <sup>1</sup>University of Connecticut, Department of Marine Sciences, 1080 Shennecossett Rd., Groton, CT 06359, <sup>2</sup>Marine Biological Laboratory, Woods Hole, MA, <sup>3</sup>Stony Brook University.

Benthic suspension-feeding invertebrates, such as bivalve molluscs, are constantly exposed to settling aggregates and the variety of microorganisms they contain. Recent research in our laboratory

demonstrates that aggregates can be a source of food particles as well as a reservoir for disease organisms. The field portion of this project is part of a large collaborative study that is addressing the ecological role of marine aggregates as a link between oysters (*Crassostrea virginica*) and the pathogen *Perkinsus marinus* and between northern quahogs (*Mercentaria mercenaria*) and the pathogen Quahog Parasite Unknown (QPX). Reference sites with low or no mortalities from these pathogens are located in Connecticut. Disease sites are located in Massachusetts and New York. At all sites, aggregate samples are collected using settling cones for the determination of the presence and concentration of pathogens. Concurrently, suspended aggregates are recorded *in situ* using a video camera focused on a stationary grid that is mounted within centimeters of the camera lens. The size and number of aggregates are determined using an image analysis program. Temperature, salinity, water depth and tidal stage are also recorded to evaluate correlations among aggregate characteristics (number and size), environmental parameters and pathogen presence. A summary of year one samples, taken from April through November, 2005 will be presented.

**AUTOGENIC ECOSYSTEM ENGINEERS AND THE INFLUENCE OF HABITAT COMPLEXITY ON INTERTIDAL MIGRATIONS BY A TRANSIENT PREDATOR.** Kirstin K. Holsman, P. Sean McDonald and David A. Armstrong. School of Aquatic and Fishery Sciences, UW.

Autogenic ecosystem engineers, which physically alter the systems they inhabit, have cascading impacts on various organisms. There are numerous examples of elevated densities within complex habitats created by native, naturalized, and exotic engineering species, yet the facilitative effects of these engineers depends on landscape patterns of their distribution as well as the size of the associated biota. Positive effects of increased physical structure often benefit small resident invertebrates while inhibiting larger transitory species. In this study, we examined how physical structure along a gradient of habitat complexity influences patterns of migration and habitat use by a transient benthic predator, Dungeness crab (*Cancer magister*). In particular, we compared crab habitat use of unstructured littoral habitats (ULH), native eelgrass beds (*Zostera marina*), naturalized Pacific oyster beds (*Crassostrea gigas*), and invasive *Spartina alterniflora* patches in Willapa Bay, WA. Baited trap surveys on ULH yielded catches of subadult *C. magister* 30–95% higher than catches from eelgrass beds, oyster beds, and meadows of *S. alterniflora*. Ultrasonic telemetry observations suggest that subadult *C. magister* making nighttime foraging incursions prefer ULH to other littoral habitats, and underwater video observations show that migrations are influenced by tidal rhythms since movements are correlated with the direction and velocity of current flow. The architecture of complex habitats

negatively influences *C. magister* utilization of littoral habitats, and ULH may be the primary foraging areas for migrating crabs. Since daily intertidal forays largely subsidize subtidal crab populations, ULH are particularly critical to crab production in these systems.

**ARE TRIPLOID HARD CLAMS RESISTANT TO FLORIDA SUMMER STRESSORS? RESULTS OF LABORATORY CHALLENGES.** Elise Hoover<sup>1</sup>, Shirley Baker<sup>1</sup>, John Scarpa<sup>2</sup> and Leslie Sturmer<sup>3</sup>. <sup>1</sup>University of Florida, Fisheries and Aquatic Sciences, <sup>2</sup>Harbor Branch Oceanographic Institution, <sup>3</sup>University of Florida, Cooperative Extension.

The hard clam, *Mercentaria mercenaria*, is an important aquaculture species in the state of Florida with \$12.9 million reported sales in 2003. Recently, clam farmers in southwest Florida have experienced high clam mortalities during the summer months. Clams may be experiencing heightened physiological stress due to an initial reduction in biomass from a spring spawning event, followed by increasing temperatures, fluctuating salinities and low dissolved oxygen levels. Triploid clams offer a potential solution to this problem as they are functionally sterile and therefore should have more energy available to survive these summer stressors. Triploidy was induced in the hard clam, *M. mercenaria*, by suppressing polar body I or II formation in fertilized eggs with cytochalasin B. Survival of triploid and diploid clams will be compared in laboratory challenges as part of a larger project to determine if there is a potential use for triploids in aquaculture. Laboratory challenges will combine typical southwest Florida conditions in a 3 × 2 factorial design. Water temperatures will be held at 32°C (90°F) in all treatments. Clams will be challenged with salinities of 15 ppt, 25 ppt, and 40 ppt and dissolved oxygen will be maintained at either normoxic or hypoxic levels. These lab-based challenge experiments will aid in determining if triploids are more stress resistant compared to diploids, thus increasing survival.

**JUVENILE CHINOOK SALMON *ONCORHYNCHUS TSHAWYTSCHA* UTILIZATION OF LOW-INTERTIDAL EELGRASS AND OYSTER AQUACULTURE BEDS.** Geoff Hosack<sup>1</sup>, Brett Dumbauld<sup>2</sup>, Ian Fleming<sup>3</sup> and David Armstrong<sup>4</sup>. <sup>1</sup>Oregon State University, <sup>2</sup>USDA-ARS, <sup>3</sup>Memorial University of Newfoundland, <sup>4</sup>University of Washington.

Estuaries function as nursery habitats for Pacific salmon populations by providing juvenile outmigrating salmonids with refugia from predators and trophic resources. Intertidal habitat in estuaries along the west coast of the U.S. is often modified by the addition of live oysters in aquaculture operations, which have become important economic contributors to small coastal economies. To as-

sess the importance of low-intertidal habitats for juvenile Chinook salmon, we compared the diet and distribution of Chinook in on-ground oyster aquaculture beds (*Crassostrea gigas*) with that in seagrass *Zostera marina* ("eelgrass") and unvegetated mudflat. We used a modified tow net to compare densities and collect diets of juvenile Chinook salmon (65–115 mm FL) in Willapa Bay, Washington. Using laboratory mesocosms, we evaluated which of these intertidal habitats are preferred by juvenile Chinook as refugia from predators. Field results suggested that outmigrating smolts did not exhibit a strong habitat preference since diet composition was unrelated to habitat type at the point of capture, and densities were significantly related to broad spatial patterns in the estuary but not habitat type within the low-intertidal zone. Older Chinook smolts (81–115 mm FL) in summer laboratory experiments significantly preferred eelgrass to mudflat or oyster in the presence of a mock avian predator while younger Chinook in spring (42–80 mm FL) demonstrated no significant change in habitat preference. This suggests that Chinook may not always exhibit strong habitat specificity, but structured intertidal habitats may provide important resources such as movement corridors on a broad landscape scale.

**STATUS, TRENDS AND RESTORATION PLANNING FOR FRESHWATER MUSSELS OF THE COLUMBIA BASIN/ PACIFIC SLOPE: THE CTUIR MUSSEL PROJECT.** Jeanette Howard, Jayne Brim Box and David Wolf. Confederated Tribes of the Umatilla Indian Reservation, 73239 Confederate Way, Pendleton, OR 97801.

The Freshwater Mussel Project of the Confederated Tribes of the Umatilla Indian Reservation (CTUIR), the first program of its kind in the West, explores new ground in the restoration of freshwater mussels. The project began in 2003, and expands on the Tribe's overall goal of recovering an intact, fully functioning, Umatilla River. Freshwater mussels were vital components of stream ecosystems in the Columbia River Basin that have been affected directly and indirectly by dams, habitat deterioration, and declines in salmon populations. Mussels were also culturally and traditionally important to Native Americans in the region. Yet, prior to this study, little was known about the distribution or status of mussel populations in the region. The overall goal of CTUIR's mussel project is to provide essential information for designing a recovery plan in the Umatilla River and other Columbia subbasins where mussels may be declining or extinct. However, this cannot be accomplished without first better understanding habitat and host fish requirements, genetic diversity, functional role, population structure and historic distribution. In this presentation, I will summarize current efforts of the CTUIR Freshwater Mussel Project to fill knowledge gaps, including: the historic and current status and distribution of freshwater mussels in CTUIR's rivers of interest; habitat variables controlling distribution; preliminary information on the functional role of freshwater mussels in these systems; host fishes and timing of reproduction. In addition, I will discuss cur-

rent efforts underway to develop an ecologically based biomonitoring program using mussels as sentinel bioindicators in the Columbia Basin.

**OREGON'S CLATSOP BEACH RAZOR CLAM FISHERIES: PAST, CURRENT AND THE FUTURE.** Matthew Hunter, Oregon Department of Fish and Wildlife.

In Oregon, the 18-mile stretch of shoreline, known as the Clatsop Beaches, extends from the South Jetty of the Columbia River south to Tillamook Head. Over 90% of Oregon's razor clam catch and effort occurs in this area. This area has had a fishery since the turn of the century and it is where the majority of biological information, recreational and commercial fishery data has been collected. Historically, the fishery has been sampled on low-tide series during the spring and summer months and as weather permitted the rest of the year. Recreational digging effort from 1995–2004 averaged 62,700 digger trips per year with a record high in 2004 of 157,000. Commercial participation from 1995–2004 averaged 125 individuals with a high of 255 individuals in 2002. The total annual harvest from 1995–2004 averaged 896,000 clams with a record harvest in 2002 of 2,660,000 clams. The Pacific razor clam is considered to be the finest food clam on the west coast resulting in the harvest being a popular tradition among coastal and inland residents. Due to this popularity, user group conflicts, harvest pressure and expectations of a sustainable fishery resource pose the continual need for the best science available to construct framework of management decisions. Many regulation changes have occurred in the past five decades as a reaction to fishery issues. With the requirement of a recreational shellfish license in 2004, funds are available and ODFW shellfish staff hope to provide proactive management to eliminate future fishery issues.

**ENVIRONMENTAL EFFECTS ON SEA URCHIN ROE ENHANCEMENT—A PIECE OF THE PUZZLE!** Phil James, National Institute of Water and Atmospheric Research, P.O. Box 14-901, Kilbirnie, Wellington, New Zealand.

The effects of various environmental conditions on the development of urchin gonads have been examined in relation to roe enhancement (fattening) of wild caught sea urchins (*Evechinus chloroticus*) in New Zealand.

The effects of wave and feeding disturbance were measured by holding urchins in sea-cages suspended from a surface line (wave disturbed) or subsurface buoyed from a bottom line (not wave disturbed) and fed and cleaned *in situ* underwater or removed from the water for feeding and cleaning. Increased water movement in the wave disturbed cages resulted in a higher GI in these urchins compared to urchins in cages that were sub surface buoyed. Feeding disturbance had no effect on the GI values or colour quality of the urchin gonads, regardless of the disturbance treatment. An experiment to measure the effects of seasonality and the initial

gonad condition on roe enhancement has shown that it is possible to significantly increase the amount of roe in a relatively short period throughout a 12 month period and that the initial condition (GI) of the urchins has a significant effect on the efficacy of roe enhancement.

**EFFECT OF DIETARY SELENIUM ON THE SEA URCHIN *LYTECHINUS VARIEGATUS*.** Warren T. Jones<sup>1</sup>, Mickie L. Powell<sup>1</sup>, Victoria K. Gibbs<sup>1</sup>, Hugh S. Hammer<sup>1</sup>, John M. Lawrence<sup>2</sup>, Addison L. Lawrence<sup>3</sup> and Stephen A. Watts<sup>1</sup>.

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Selenium is an essential micronutrient in the diet of many animals; however, high levels may be toxic. In mammals, selenium is an important antioxidant, working with vitamin E to protect cell membranes and prevent free radical generation. Small *L. variegatus* (ca.15.5 g initial wet weight) were fed formulated feeds with low, medium, and high levels of selenium for 12 weeks (calculated at 0.23, 0.91, and 2.5 ppm; levels based on previously established dietary levels for other marine invertebrates, supplemented as Na<sub>2</sub>SeO<sub>3</sub>). Consumption was measured daily, and urchins were weighed and test diameters measured at 4-week intervals. Wet weights of individuals fed the high selenium feed were 12% lower ( $p = 0.006$ ) than those fed the low and medium levels ( $43.11 \pm 1.21$ ,  $43.03 \pm 1.22$ , and  $37.81 \pm 1.37$  g wet weight for low, medium, and high, respectively) of dietary selenium at 12 weeks. Urchins fed the low selenium feed had higher specific growth rates between four and eight weeks (1.5 vs. 1.2 and 1.1% body wet weight gain/day for low, medium and high levels, respectively). Additionally, test diameters for urchins fed the low and medium selenium feeds were slightly higher ( $p = 0.077$ ) than those fed the high selenium ( $43.2 \pm 0.39$ ,  $43.2 \pm 0.50$ , and  $41.8 \pm 0.51$  mm, respectively) feed. Feed consumption rates were similar among treatments suggesting weight gain was affected by other physiological mechanisms. Although no mortalities were observed, noticeable pathologies were found in the urchins fed high selenium feeds. These results suggest dietary toxicity at high levels of selenium.

**DEVELOPMENT OF SINGLE NUCLEOTIDE POLYMORPHISMS (SNPS) IN *CRASSOSTREA ARIAKENSIS* AND RELATED *CRASSOSTREA* SPECIES.** Hyungtaek Jung<sup>1</sup>, Woojin Kim<sup>2</sup> and Patrick Gaffney<sup>1</sup>. <sup>1</sup>University of Delaware, Graduate College of Marine Studies, Lewes, DE 19958, <sup>2</sup>National Fisheries Research and Development Institute.

As a candidate for deliberate introduction into Atlantic waters, the Suminoe, or jinjiang oyster, *Crassostrea ariakensis*, has recently been the subject of intense interest, with particular focus on its ecology, taxonomy and population genetics. Because the tax-

onomy of the Asian *Crassostrea* is still incomplete and identification on morphological grounds is difficult, a suite of genetic markers suitable for identifying species and subspecies is needed. Such markers will allow screening of candidate source material, allow development of genetic signatures of hatchery strains for evaluating introductions, and ultimately provide tools for selective breeding and pedigree monitoring. We have tested primers originally designed to amplify fragments of known genes (Type I markers) in *C. gigas* for their ability to amplify putative homologs in the related Asian species *C. angulata*, *C. ariakensis* and *C. hongkongensis*. Of 16 loci developed for *C. gigas*, all amplified successfully in *C. angulata*, 15 amplified in *C. ariakensis* and 14 amplified in *C. hongkongensis*. In contrast, only five loci could be amplified in the Atlantic species *C. virginica*. Direct sequencing of amplicons revealed multiple intraspecific and interspecific candidate polymorphisms, and provided tools for genetic identification, linkage mapping, pedigree monitoring and phylogenetic analysis.

**INGESTION OF MARINE AGGREGATES BY SUSPENSION-FEEDING MOLLUSCS.** Dustin Kach and J. Evan Ward. University of Connecticut, Department of Marine Sciences, 1080 Shennecossett Rd., Groton, CT 06359.

A large body of literature focuses on suspension-feeding processes of molluscs, including the efficiency of particle capture. Bivalves, for example, capture individual picoplankton cells (0.2–2.0  $\mu\text{m}$ ) with a retention efficiency of less than 50%, leading to the assumption that such particles are not an important food resource. Picoplankton, however, are often concentrated within aggregates of much larger size. This study builds on our preliminary results and further investigates the ability of suspension feeders to ingest pico-particles (<2  $\mu\text{m}$ ) bound in aggregates. We fed the mussel, *Mytilus edulis*, the scallop, *Argopecten irradians*, and the slipper snail, *Crepidula fornicata*, 1  $\mu\text{m}$  fluorescent beads that were (1) evenly dispersed in seawater, or (2) embedded within laboratory-made aggregates. Dispersed 10  $\mu\text{m}$  beads were also delivered so that feeding activity could be determined. All feces were collected over 48 hr period, digested in sodium hydroxide (NaOH<sup>+</sup>), and the fluorescent activity measured by a spectrophotometer. The number of beads ingested was then calculated using a previously established standard curve. Results indicate that aggregates significantly enhance the ingestion of 1  $\mu\text{m}$  beads by all three animals. Differences among species in their ability to ingest aggregates and pico-particles, however, were evident. When feeding on aggregates, mussels demonstrated a ten fold increase in the number of 1  $\mu\text{m}$  beads ingested. Compared to mussels, scallops ingested fewer aggregates, and slipper snails ingested more dispersed beads. These differences may be a consequence of variations in gill structure and mechanisms of particle processing. Our data suggest that aggregates allow suspension feeders to utilize embedded particles as food.

**IDENTIFICATION OF GROWTH BANDS IN COCKLE, *FULVIA MUTICA* SHELLS IN KOREA.** Do-hyung Kang<sup>1</sup>, Heng-sik Park<sup>2</sup>, Chris A. Richardson<sup>3</sup> and Kwang-sik Choi<sup>1</sup>.

<sup>1</sup>Cheju National University, College of Marine Science, School of Applied Marine Science, 1 Ara 1 Dong, Jeju City, Jenu-Do 690-1786 Republic of Korea. <sup>2</sup>Korea Ocean Research & Development Institute, <sup>3</sup>University of Wales, UK.

This study reports identification of annual growth bands recorded in shell from cockle, *F. mutica* (Bivalvia: Cardiidae) collected from Jukdo, Korea. The growth lines were identified using acetate peel replicas of the shell sections. Total number of growth lines and distance (i.e. growth interval) between inter-growth lines were assessed using image-processing software. Age of the cockle was estimated by counting the number of each dark band in sectioned shell and umbo. Periodicity of the light and dark bands was compared with the daily water temperature data of the sampling area retrospectively. A total of 790 growth lines were identified from the umbo. Age of the cockle used in the analysis was predicted to be 6.5 year. The maximum distance (23.4  $\mu\text{m}$ ) and the minimum distance (1.2  $\mu\text{m}$ ) were found in a 3-year-band and in a 6-year-band, respectively. Average distance of the cockle was highest in 3-year-band. Periodicity of the bands was closely corresponded with temperature data, suggesting that retrospective technique was suitable for aging of the cockle.

**MICROGROWTH BANDING PATTERNS IN THE SHELL OF THE COCKLE, *FULVIA MUTICA* FROM KOREA.** Do-Hyung Kang<sup>1</sup>, Heng-Sik Park<sup>2</sup>, Chris A. Richardson<sup>3</sup> and Kwang-Sik Choi<sup>1</sup>.

<sup>1</sup>School of Applied Marine Science, College of Ocean Science, Cheju National University, 1 Ara 1-Dong Jeju 690-756 Republic of Korea, <sup>2</sup>Korea Ocean Research & Development Institute, Ansan P.O. Box 29 425-600 Republic of Korea, <sup>3</sup>School of Ocean Sciences, University of Wales, Bangor, Menai Bridge, Gwynedd LL59 5AB, UK.

Growth banding, consisting of a pattern of narrow tidally deposited dark bands and wider light growth increments, were identified in acetate peel replicas of shell section of live collected cockle, *Fulvia mutica* (Bivalvia: Cardiidae) collected subtidally from Jukdo, Korea. The total number of dark growth bands and the inter-band distance (i.e. growth increments) were counted and measured respectively using image-processing software. The age of the cockle was estimated by counting the number of dark bands in the sectioned shell and the prominent dark lines in the umbo region. The oldest cockle determined using these methods was 6.5 years. The width of the growth increments varied from 23.4  $\mu\text{m}$  in a 3rd dark line to 1.2  $\mu\text{m}$  in a 6th dark line in the umbo region of oldest cockle. Each wide light tidal increment was dated and the width of the increment compared with the daily seawater temperature; a positive correlation between increment width and seawater

temperature was observed opening up the possibility of using the tidal increments for environmental reconstruction of shell growth rates.

**APPLICATION OF RECENT RED ABALONE *HALIOTIS RUFESCENS* SURVEYS TO MANAGEMENT DECISIONS OUTLINED IN THE CALIFORNIA ABALONE RECOVERY AND MANAGEMENT PLAN.** Jerry V. Kashiwada<sup>1</sup> and Jan K. Taniguchi<sup>2</sup>. <sup>1</sup>California Department of Fish and Game, Fort Bragg, CA 95437, <sup>2</sup>California Department of Fish and Game, Los Alamitos, CA 90720.

The management section of California's recently adopted Abalone Recovery and Management Plan (ARMP) uses results of fishery independent transect surveys at eight index sites to regulate Total Allowable Catch (TAC). A decision table in the ARMP (Table 1) uses densities in deep water (>8.2 to 19.7 m), densities over all depths, and successful recruitment (>4,500 abalone/hectare in the 100–177 mm size class) to decide whether changes need to be made in the TAC. The TAC is estimated from telephone surveys and returned Abalone Permit Report Cards. Recent surveys in 2003 and 2005 at four of the eight index sites show red abalone densities in the range of the baseline densities established by surveys in 1999 and 2000. Applying the results of the 2003 and 2005 surveys to the ARMP decision table indicates no change is needed in the current TAC. Two-way ANOVA found there was no difference in density between sites and time periods for all depths at two of the sites (Van Damme and Salt Point) which were surveyed in both time periods. Likewise there was no significant difference at deep depths between these two sites and time periods.

**COULD *LIMNOPERNA FORTUNEI* BE WORSE THAN *DREISSENA POLYMORPHA*? POTENTIAL SPREAD AND ECOSYSTEM IMPACTS.** Alexander Y. Karatayev<sup>1</sup>, Demetrio Boltovskoy<sup>2</sup>, Dianna K. Padilla<sup>3</sup> and Lyubov E. Burlakova<sup>1</sup>. <sup>1</sup>Stephen F. Austin State University, Department of Biology, Box 13003-SFA Station, Nacogdoches, TX 75962-3003, <sup>2</sup>Universidad de Buenos Aires, Argentina, <sup>3</sup>Stony Brook University, Stony Brook, NY.

Although taxonomically unrelated, the zebra mussel (*Dreissena polymorpha*) and golden mussel (*Limnoperna fortunei*) have very similar life histories, suggesting that similar vectors are involved in their spread. Both are sessile, byssate bivalves with a planktonic larval stage and high reproductive capacity. Adults of both species attain extremely high densities physically changing the substrate, and because they are such effective suspension feeders they greatly enhance benthic-pelagic coupling and act as powerful ecosystem engineers. We compare environmental limits and ecological impacts of the well studied *D. polymorpha* with the lesser known *L. fortunei* to predict the potential spread and ecosystem impacts of the golden mussels. *Limnoperna fortunei* reaches population densities similar to or higher than *D. polymorpha*. It has comparable

filtering rates and therefore may have similar or even stronger ecosystem impacts. In addition, *L. fortunei* has broader environmental tolerances than *D. polymorpha*, including higher water temperatures, lower pH levels, lower calcium content and water pollution. Therefore, *L. fortunei* may be much more successful than *D. polymorpha* in regions dominated by acidic, soft and contaminated waters. We predict that in the near future *L. fortunei* will colonize the southern and central parts of North America, Southern Europe, Northern Africa, and Australia, as well as expand its distribution in South America and Asia. Although to date *D. polymorpha* is considered the most aggressive freshwater invader, soon many waterbodies that are already affected by *D. polymorpha* may get another, even more aggressive invader.

**ENHANCED BLUE CRAB ABUNDANCE ON RESTORED OYSTER REEFS.** M. Lisa Kellogg<sup>1</sup>, Christopher McIntyre<sup>2</sup>, Kennedy C. Paynter<sup>1</sup> and Kennedy T. Paynter<sup>1</sup>. <sup>1</sup>University of Maryland, <sup>2</sup>Coastal Carolina University.

In recent years, the goals of oyster reef restoration in Chesapeake Bay have shifted from simply enhancing the oyster fishery to restoring the ecosystem services that were once provided by healthy oyster reefs. Like historical reefs, restored oyster reefs produce topographically-complex reef structures that enhance both the abundance and diversity of the associated macrofaunal community. Restored oyster reefs, therefore, have the potential to provide highly-mobile organisms such as the blue crab, *Callinectes sapidus*, with both an abundant supply of prey and a refuge from predation. To determine whether restored oyster reefs are a preferred habitat for blue crabs, blue crab populations were estimated on restored oyster reefs and adjacent control areas at two restoration sites in the South River: Glebe Bay and Duvall Creek. On each sampling day, one 50-m trotline was laid on the restored reef and another was placed on an adjacent unrestored control area. Alternating between the two lines, each line was sampled 10 times. All crabs caught were retained to avoid recapture of the same individuals. More crabs were caught on the restored reefs than the control sites at both Glebe Bay ( $p = 0.004$ ) and Duvall Creek ( $p = 0.037$ ). Assuming these capture rates represent relative crab abundances, these preliminary findings suggest that blue crab densities may be higher on restored oyster reefs. However, additional studies will be necessary to determine why blue crabs prefer the restored reefs, and whether restored reefs enhance blue crab production.

**DEVELOPMENT OF SINGLE NUCLEOTIDE POLYMORPHISMS (SNPS) IN THE PACIFIC OYSTER *CRASSOSTREA GIGAS*.** Woo-jin Kim<sup>1</sup>, Hyoungtaek Jung<sup>2</sup> and Patrick Gaffney<sup>2</sup>. <sup>1</sup>National Fisheries Research and Development Institute, Busan, South Korea, <sup>2</sup>University of Delaware.

Single nucleotide polymorphisms (SNPs) are useful for characterizing population structure, genetic mapping, and as a tool for pedigree monitoring and marker-assisted breeding schemes. Our

objective is to develop SNP markers for the Pacific oyster *Crassostrea gigas*, the most widely cultured bivalve species. Primers were designed based from expressed sequence tags (ESTs) to amplify small fragments (200–400 bp) spanning the 3' end of coding sequence and the 5' end of the 3'UTR. Most (22 of 24) primer pairs successfully amplified PCR products from genomic DNA. Direct sequencing of amplified targets resulted in the identification of 321 candidate SNPs within 22 identified nuclear genes. The observed frequency of SNPs in *C. gigas* was 4.32 per 100 bp: 1.94 in exons, 9.06 in introns, and 4.64 in the 3'UTR. Individuals from an inbred hatchery line showed reduced polymorphism. Oysters from three populations of *C. gigas* (Korea, Hokkaido, Kyushu) and one *C. angulata* population (Portugal) could be differentiated using estimated genetic similarity based on SNP allele-sharing at ten loci. All loci were successfully amplified from *C. angulata*, underscoring its close genetic relationship to *C. gigas*. The majority of identified SNPs differed between the two species, providing multiple independent markers for identification of both species and hybrids, and for marker-assisted introgressive hybridization. Mapping of these loci is in progress. The SNP markers developed in this study will enable researchers to elucidate population structure in the native range of the two species, as well as the characterization of cultured lines.

**RELATIONSHIPS AMONG PARASITES AND PATHOLOGIES IN SENTINEL BIVALVES: NOAA STATUS AND TRENDS 'MUSSEL WATCH' PROGRAM.** Yungkul Kim and Eric N. Powell, Haskin Shellfish Research Laboratory, Rutgers, The State University of New Jersey, 6959 Miller Ave., Port Norris, NJ 08349 USA.

NOAA's National Status and Trends 'Mussel Watch' Program includes a comprehensive survey of the histopathology of sentinel bivalves from the East, West, Gulf, and Great Lakes coasts of the United States. We analyzed the data for 1995–1998 to identify relationships between various parasites, various pathologies, and between parasite/pathology pairs with the goal of identifying consistencies and differences in these relationships between sentinel bivalves and between major geographic units of the U.S. coastline. The prevalences of parasite, pathology, and parasite-pathology pairs were significantly correlated more frequently for oysters than for mussels. The number of significant correlations within Gulf-coast oysters exceeded the number within East-coast oysters. Correlations were least frequent among East-coast mussels. The incidence of significant negative correlations in prevalence far exceeded the incidence of significant positive correlations in all species and bay regions. Significant relationships in infection intensity occurred much less frequently than for prevalence. Positive correlations occurred more frequently than they did for prevalence. Both trends reinforce the belief that environmental factors controlling transmission are likely distinctive from those controlling proliferation. Only a few relationships between parasites were com-

mon to more than one sentinel bivalve or more than one coastal region. Not a single common relationship involved a pathology. However, though commonalities were few, consistent trends in prevalence between mussels and oysters and between coastal regions suggest potentially important large-scale trends among some important parasite groups, particularly the gregarines and gut ciliates, gill gregarines and cestodes, prokaryotic inclusions and trematode metacercariae, and *Perkinsus marinus* and the gregarines.

**COMPARISONS OF POST-SETTLEMENT SURVIVAL AND GROWTH IN *CRASSOSTREA VIRGINICA* AND *C. ARIAKENSIS* IN RELATION TO TIDAL EMERSION.** Peter R. Kingsley-Smith and M. Luckenbach, Virginia Institute of Marine Science.

In many high salinity regions of the mid-Atlantic *C. virginica* is limited to the intertidal zone where it achieves a partial refuge from predation. Persistence in such intertidal habitats necessitates tolerance of both desiccation and extremes of temperature during periods of aerial exposure. Such capabilities in *C. ariakensis* have yet to be determined. The effects of aerial exposure by tidal emersion on comparative survival and growth rates of diploid *C. ariakensis* and diploid *C. virginica* were investigated using oysters set on plastic tiles and grown in a flow-through quarantine system. Four tidal regimes were simulated: 1) *high intertidal* (3.5 hrs emersion), 2) *mid tide* (2 hrs emersion), 3) *low intertidal* (1 hr emersion) and 4) *subtidal* (constant immersion). Vertical ("North-facing" and "South-facing") and horizontal ("Up" and "Down") tile orientation treatments were also incorporated in the experimental design. Tiles were individually photographed on a weekly basis between June and August 2005 and image analysis software was used to gather survival and growth data.

The high intertidal treatment exceeded the physiological tolerances of both species; complete mortality occurred within the first two weeks. In the mid tide and the low intertidal treatments *C. ariakensis* and *C. virginica* exhibited intermediate growth and survival. Growth rates were highest and levels of mortality were lowest in the subtidal treatment. Determination of the tolerance of *C. ariakensis* to aerial exposure will improve our understanding of its potential to colonize intertidal habitats, to compete with native species for resources, and to become a fouling nuisance if introduced.

**MEASUREMENT OF SCOPE-FOR-GROWTH IN FRESHWATER MUSSELS AND THE RELEVANCE FOR WATER QUALITY, ECOSYSTEM FUNCTION AND BIOMONITORING.** Danielle A. Kreeger, Partnership for the Delaware Estuary, 110 S. Poplar St., Suite 202, Wilmington, DE 19801.

Native freshwater mussels continue to garner increasing attention for their biodiversity plight, ecological value, and usefulness for biomonitoring. Despite the rising chorus, many key aspects of

their basic biology remain poorly studied. Of particular note is the lack of attention to organism-level physiological functions that represent the nexus, or "missing link," essential to relating environmental conditions to population- and species-level fitness, and vice versa. Small spatial and temporal shifts in environmental conditions, food quantity and quality, or animal nutritional demands can elicit significant shifts in organism-level processing rates and fitness, with concomitant effects to the ecosystem where populations remain abundant. Decades of research with marine species have yielded an arsenal of powerful methods to quantify spatial, temporal, intraspecific and interspecific variation in physiological rates and status. And yet, only one physiological rate metric (clearance) and one status metric (condition) have been widely adopted for freshwater mussels, and their meaning has at times been misinterpreted. In this presentation I will briefly review the available "tool kit," describe methods needed to calculate scope-for-growth, and discuss the proper interpretation of these and other physiological metrics as being context specific, rather than absolute. Specifically, I will present case study data for the allometric rates of clearance, defecation, excretion and respiration of species from both the Atlantic and Pacific slopes. When standardized and understood, physiological methods and information will play a pivotal role in monitoring and optimizing the health of captive animals, quantifying the functional importance of mussel populations in nature, and biomonitoring the status of our waterways.

**TRANSPARENT EXOPOLYMER PARTICLES (TEP) FROM SUSPENSION FEEDERS AND THEIR EFFECTS ON PARTICLE AGGREGATION.** Binglin Li and J. Evan Ward, University of Connecticut, Marine Sciences Building, 1080 Shennecosset Rd., Groton, CT 06340.

Transparent exopolymer particles (TEP) can form from dissolved precursors released by phytoplankton. Recent studies have also demonstrated that benthic suspension feeders, such as bivalves, can contribute significantly to the TEP pool in coastal waters. Whereas TEP from phytoplankton is important for the aggregation of suspended material, the role of TEP from suspension feeders on aggregate formation is unknown. The purpose of this study was to test the hypothesis that organic compounds, including TEP, released from suspension feeders enhance the formation of particle aggregates in coastal waters. In laboratory experiments, TEP was collected from blue mussels (*Mytilus edulis*) and sea vases (*Ciona intestinalis*) using previously established methods. One of two particle types, silica-amino beads or diatomaceous earth (ca. 10  $\mu\text{m}$ ), were then added to seawater with and without TEP produced by the animals. Bottles (250 ml) containing suspended particles were placed on a roller table and rolled for 48



hours. Changes in the size distribution of particles in each jar were followed over time by means of an electronic particle counter, and aggregates larger than 100  $\mu\text{m}$  were counted using a rafter cell and microscope. Dissolved and total organic carbon and bacterial concentrations were determined before and after the rolling period. Results indicate that significantly more particle aggregates are produced in seawater containing TEP from suspension-feeding animals than in control water. Aggregation of both particles types was similarly enhanced. Our results suggest that organic material released by suspension-feeders can enhance aggregate formation and hence the deposition of material to the benthos.

**VARIANCE IN REPRODUCTIVE SUCCESS OF THE FLAT OYSTER *OSTREA EDULIS* L. ASSESSED BY MICROSATELLITE-BASED PARENTAGE ANALYSES.** Delphine Lallias<sup>1</sup>, Nicolas Tairs<sup>1</sup>, Pierre Boudry<sup>1</sup>, François Bonhomme<sup>2</sup> and Sylvie Lapègue<sup>1</sup>. <sup>1</sup>INFREMER—LGP, Station de La Tremblade, La Tremblade, France 17390, <sup>2</sup>UMR5171 Ifremer-CNRS—Université Montpellier II, Sète, France.

The European flat oyster (*Ostrea edulis* L.) is a marine bivalve whose natural geographical distribution ranges along the European Atlantic coast from Norway to Morocco, in addition to the Mediterranean and Black Sea. Previous studies of allozymes, microsatellites and mitochondrial differentiation over the whole range concluded that a significant divergence existed between Mediterranean and Atlantic populations, together with an isolation-by-distance pattern. However, the average mitochondrial haplotypic diversity displayed a high among populations variance, reflecting smaller effective population size in some locations. Additionally, a ten-fold quantitative difference was observed in the same study in Fst between the mitochondrial and the nuclear genomes, which could be due to sex biased differential reproductive success between males and females. In order to further document this hypothesis, two experiments were conducted. First, brooding females were sampled in a wild population and the number of males fertilizing a given female estimated. Then, parentage analyses were achieved under experimental conditions: successive mass spawnings were collected from a population of potential genitors kept in hatchery, whose genotypes were known, in order to infer a posteriori the relative contribution of each genitor. Moreover, we aimed to better understand the reproduction dynamics of this species. Several patterns of spawning could be distinguished: unique, successive or extended in time. The different parental contributions and reproductive behaviors observed in these experiments are discussed in the context of the hypothesis of a variance in the reproductive success of males and females and consequences in local and temporal reduced effective population sizes.

**TRANSCRIPTOME ANALYSIS OF PACIFIC OYSTER *CRASSOSTREA GIGAS* FAMILIES EXPOSED TO HEAT STRESS.** Paul Lang<sup>1</sup>, Christopher J. Langdon<sup>1</sup> and Mark D. Camara<sup>2</sup>. <sup>1</sup>Oregon State University, Department of Fisheries and Wildlife, Hatfield Marine Science Center, Newport, OR 97365, <sup>2</sup>United States Department of Agriculture, ARS Program, Hatfield Marine Science Center, Newport, OR 97365.

We studied the transcriptome level response of oysters to heat stress using microarrays developed by the Oyster Microarray Consortium to compare gene expression among selectively-bred oyster families that differed in sensitivity to heat stress. Spat ( $n = 100$  each per family) of 53 oyster families were exposed to 43°C water for 1 h. We then assessed mortality 2<sup>nd</sup> intervals for six d. Survival ranged from 6% to 85% and we classified families with >70% survival as resistant those with 30–70% survival as moderately resistant, and those with <30% survival as sensitive. Animals from the four highest- and the four lowest-surviving families were reared to two years of age and tested again. Survival ranged from 0.03% to 95% for adults. Four of the eight families retained their classification of heat-resistant or heat-sensitive, and the remaining four families were re-classified as moderately heat sensitive.

For transcriptome analysis, we exposed the two resistant families and two susceptible families to a non-lethal heat shock (40°C for 1 h) and collected gill tissue from three animals per family at 1 h, 3 h, 6 h, and 24 h following heat shock. We pooled tissue samples within family for each sampling time and extracted total RNA from each pooled sample. We labeled each RNA sample with a fluorescent dye, and hybridized the sample to a single microarray chip. Fluorescence data was collected using a Scan-Array Express instrument. Genes that underwent coordinated changes in expression level were clustered into groups using multivariate exploratory analysis. The results of these analyses will be discussed.

**THE POTENTIAL ROLE OF FRESHWATER MUSSELS IN REDUCING *GIARDIA* AND OTHER HUMAN PATHOGENS IN WATERSHEDS OF THE PACIFIC NORTHWEST.** Chris Langdon<sup>1</sup>, David Close<sup>2</sup> and Peggy Bradley<sup>3</sup>. <sup>1</sup>Hatfield Marine Science Center, Oregon State University, <sup>2</sup>Confederated Tribes of the Umatilla Indian Reservation, <sup>3</sup>Institute of Simplified Hydroponics.

Freshwater mussels were abundant in rivers of the Pacific Northwest and were an important source of nutrition for many Native Americans in pre-European times. Since then, populations of freshwater mussels have declined. Habitat changes, pollution and declines in salmon (an important upstream transport vector of larval stages) are likely causes. These declines will result in a reduction in the filtering capacity of mussel populations and removal of suspended sediments and organisms, including the human pathogens *Giardia duodenalis* and *Cryptosporidium parvum*.



Laboratory studies showed that adult (91–127 mm shell length) freshwater mussels (*Margaritifera falcata*) filtered mice-derived *Girardia* (*G. muris*) at rates of  $0.49 \text{ l h}^{-1} \text{ g}^{-1}$  mussel dry weight. In addition, mussels filtered microalgae [*Schizochytrium* sp.; in the same size range as *G. duodenalis* (2.7–7.1  $\mu\text{m}$ )] at rates of 0.39 to  $0.41 \text{ l h}^{-1} \text{ g}^{-1}$  dry weight. The filtering action of mussels could be an important natural mechanism for reducing concentrations of human pathogens in streams and rivers.

**IMPROVEMENT IN YIELDS OF THE PACIFIC OYSTER *CRASSOSTREA GIGAS* AFTER TWO GENERATIONS OF SELECTION.** Chris Langdon, Ford Evans, Sean Matson, Drew Mosher and Alan Barton. Hatfield Marine Science Center, Oregon State University.

Globally, the Pacific oyster is the most valuable aquaculture species, with a harvest of 4.2 million metric tons valued at \$3.5 billion (FAO Fisheries Statistics 2002). The West Coast is the biggest regional producer of oysters in the U.S. with a wholesale value of \$68 million. In contrast to many other U.S. agricultural commodities, there had been no long-term, funded research program to select and manage Pacific oyster broodstock for enhanced production. In response to this need, the Molluscan Broodstock Program (MBP) was established in 1995 to implement a selective breeding program to improve broodstock and increase commercial production of Pacific oysters on the West Coast, U.S. Since MBP's inception, about 1300 oyster families have been planted and evaluated at commercial grow-out sites from Alaska to California, U.S. Results show that after two generations of selection, the average yield of oyster families derived from selected MBP broodstock is 29% greater than that of families from 'wild' unselected broodstock and 34% greater than that of industry broodstock. Furthermore, the five top-performing families (recommended for commercial hatcheries) from F2 MBP cohorts have an average yield that is 77% greater than that of families from industry broodstock. MBP broodstock are used in commercial hatcheries for large-scale seed production.

**A LEAST COST FORMULATED FEED FOR GONAD PRODUCTION OF THE SEA URCHIN *LYTECHINUS VARIEGATUS*.** A. L. Lawrence<sup>1</sup>, S. A. Watts<sup>2</sup>, M. L. Powell<sup>2</sup> and J. M. Lawrence<sup>3</sup>. <sup>1</sup>Texas A&M University System, 1300 Port Street, Port Aransas, Texas 78373, <sup>2</sup>University of Alabama, Birmingham, Birmingham, Alabama 35294, <sup>3</sup>University of South Florida, Tampa, Florida 33620.

Feeds are critical to successful aquaculture production. Feed usually represent 30% to 50% of the variable cost. Dry pelleted semi-purified feeds have recently been used to produce roe of marketable quality for *Lytechinus variegatus*. Using ingredient and nutrient limits for semi-purified feeds and substituting practical

ingredients for purified ingredients such as whole wheat grain for wheat starch and dehulled, defatted soybean meal (48% protein) for purified isolated soy protein, a commercial urchin feed was formulated using a least cost program. Calculated feed levels for crude protein, soluble carbohydrate, crude fiber, total ash and crude fat were 25%, 34.9%, 2.9%, 19.5%, and 7.5%, respectively, with an ingredient cost of \$609.90/M.T. Protein was the most expensive nutrient with feed costs decreasing to \$557.70 and increasing to \$659.80/M.T. for 15% and 35% protein feed levels, respectively. Marine (e.g. kelp, fish) and lipid (e.g. carotenoid, cholesterol) ingredients represented the largest ingredient costs. Estimating feed mill administrative, amortization, production and margin is approximately \$200/M.T., the selling price for 25% protein commercial urchin feed would be \$809.90/M.T. Using shrimp raceway and feed technology and economic data and data obtained last year by the University of Alabama at Birmingham and Texas A&M System, urchin aquaculture for roe production for human consumption has a larger potential margin than intensive shrimp production in raceways.

**SIZE-SPECIFIC SURVIVAL AND FISHING MORTALITY ESTIMATES FOR RED ABALONE, *HALIOTIS RUFESCENS*, USING MARK-RECAPTURE DATA.** Robert Leaf<sup>\*1</sup>, Laura Rogers-Bennett<sup>2</sup> and Peter L. Haaker<sup>3</sup>. <sup>1</sup>Virginia Tech, <sup>2</sup>Bodega Marine Lab and California Department of Fish and Game, <sup>3</sup>California Department of Fish and Game.

Estimates of size specific natural and fishing mortality are currently not available for red abalone, *Haliotis rufescens*, in northern California. We examined annual survivorship of three size classes (<100 mm, 100.1 to 178 mm, and >178 mm, the legal fishing limit) of red abalone using capture-mark-recapture data in northern California. We estimate fishing mortality comparing fished and reserve sites in northern California. The number of tagged individuals ( $n = 273$  to 2,145), survey occasions ( $n = 3$  to 7), and size composition of individuals (41.5 to 227 mm, maximum shell length, MSL) were variable at each site. The annual survival probabilities of the smallest size class (<100 mm MSL) at one reserve site in northern California was  $0.52 \text{ y}^{-1} \pm (0.05 \text{ SE})$  and was  $0.36 \text{ y}^{-1} \pm (0.07 \text{ SE})$  to  $0.51 \text{ y}^{-1} \pm (0.08 \text{ SE})$  in southern California. Annual survival of the mid-size class (100.1 to 178 mm MSL) from four sites at northern California ranged from  $0.47 \text{ y}^{-1} \pm (0.05 \text{ SE})$  to  $0.71 \text{ y}^{-1} \pm (0.04 \text{ SE})$ . The largest size class (>178 mm MSL) had a large range of annual survivorship values of  $0.26 \text{ y}^{-1} \pm (0.06 \text{ SE})$  to  $0.95 \text{ y}^{-1} \pm (0.08 \text{ SE})$  in fished and reserve sites in northern California. Instantaneous fishing mortality ( $F$ ) was estimated to be 0.68 to 1.29. This study is an example of how capture-mark-recapture data from fished and reserve sites and the selection of parsimonious models can be used to estimate natural and fishing mortality estimates.

**PRELIMINARY VALIDATION OF AGE-AT-LENGTH OF RED ABALONE, *HALIOTIS RUFESCENS*, USING BOMB RADIOCARBON.** Robert T. Leaf<sup>1</sup>, Allen H. Andrews<sup>2</sup> and Gregor M. Cailliet<sup>2</sup>. <sup>1</sup>Virginia Tech, <sup>2</sup>Moss Landing Marine Laboratories.

Current methods to describe the age-at-length relationship of red abalone (*Haliotis rufescens* Swainson) are insufficient to determine the ages of large individuals because their shell lengths exceed those predicted by model  $L_{\infty}$  values. We evaluated the utility of analyzing atomic bomb generated radioisotope  $^{14}\text{C}$  in shell as a method to validate the age-at-length relationship of *H. rufescens*. Fabens' (1965) method was used to determine von Bertalanffy growth function (VBGF) parameters ( $L_{\infty}$  313 mm,  $k = 0.051 \text{ y}^{-1}$  (0.042 to 0.059, 95% CI)) based on data from a multi-year, multi-site tag-recapture study. Shell carbonate was sampled at four locations on a single shell (251 mm, maximum shell length (MSL)) with the objective to bracket the rise of the radiocarbon signal, known to occur during ~1957 in the NE Pacific. There was close correspondence to the radiocarbon values of extracted shell carbonate and estimated dates of formation based on VBGF estimates. The 251 mm (MSL) red abalone specimen was predicted to be 27 to 38 years old. This study presents preliminary results from radiocarbon analysis of shell carbonate and demonstrates the utility of validating the age-at-length relationship of red abalone with this method.

**SEED QUALITY ASSESSMENT OF *MYTILUS EDULIS* STOCKS IN THE GULF OF ST. LAWRENCE, CANADA.** Neil Leblanc<sup>1</sup>, Fabrice Pernet<sup>1</sup>, Rejean Tremblay<sup>2</sup>, Thomas Landry<sup>3</sup> and Jeff Davidson<sup>4</sup>. <sup>1</sup>Coastal Zones Research Institute, <sup>2</sup>University of Quebec, <sup>3</sup>Fisheries and Oceans, <sup>4</sup>Atlantic Veterinary College.

Seed supply is a crucial part of any aquaculture operation and the mussel industry in eastern Canada is no exception. This industry relies solely on wild spat collection to furnish seed for its aquaculture operations. High mortality in cultured mussels has periodically been a problem in the Gulf region and this has sometimes been attributed directly to the seed stock that has been used. This has occurred in the absence of a pathological explanation and studies on mussel stocks in the Gulf region have pointed to the genetic composition of mussel populations as a significant factor contributing to large scale mortality events.

The objective of the study was to develop and assess seed quality criteria for *Mytilus edulis*. We have taken mussel seed from six different locations in two provinces in the Gulf region and performed a reciprocal transfer among the seed source sites creating a 6 × 6 (seed × location) field study using a total of 3240 individually tagged mussels. Productivity was measured using the variables growth, survival, and condition index. Explanatory vari-

ables included lipid content and composition, heterozygosity level, microsatellites loci characterization, histology and oxygen consumption.

**THE EFFECTS OF ANTI-FOULING TREATMENTS FOR *STYELA CLAVA* ON LONG-LINE CULTURED *MYTILUS EDULIS* IN PRINCE EDWARD ISLAND, CANADA.** Neil Leblanc<sup>1</sup>, Jeffery Davidson<sup>1</sup>, Mary McNiven<sup>1</sup>, Thomas Landry<sup>2</sup> and Rejean Tremblay<sup>3</sup>. <sup>1</sup>Atlantic Veterinary College, <sup>2</sup>Fisheries and Oceans, <sup>3</sup>University of Quebec.

Currently, the mussel industry in Prince Edward Island is facing serious production problems associated with an invading species, *Styela clava*, or clubbed tunicate, is an invading species found in Prince Edward Island in the late 1990's. It is a sessile filter feeder, which forms dense colonies on mussel lines causing several serious production problems. In the first few bays found to contain this organism, the mussel farms were overwhelmed by this invader. The objective of this experiment was to determine the effect that acetic acid treatments, used to get rid of the tunicates, has on the mussels being farmed. A field trial was conducted in November 2002 involving 1/2 inch socked juvenile mussels. The trial involved two acetic acid treatments, a thirty second and two-minute dip in 5% acetic acid. The treated and control socks were then placed systematically on a longline in New London Bay, Prince Edward Island from north to south in the order two-minute acetic acid dip, control, thirty second acetic acid dip. Seven months later, in June 2003, the line was retrieved. Studies in the lab consisted of weighing the entire sock, two-foot sections, measuring 500+ specimens per treatment for length, performing a condition index experiment on thirty specimens of each treatment, and allozyme analysis on fifty individuals per treatment to determine heterozygosity. This experiment indicates that acetic acid can be fatal to mussels but does not cause serious longterm health problems. These factors should be taken into account when devising methods and dosages for the anti-fouling treatments.

**MINING EST DATABASE FOR SINGLE-NUCLEOTIDE POLYMORPHISMS IN THE EASTERN OYSTER (*CRASSOSTREA VIRGINICA*).** Jeong-ho Lee and Ximing Guo. Haskin Shellfish Research Laboratory, Rutgers University.

Single-nucleotide polymorphisms (SNPs) are important resources in genomic analysis, particularly when they are derived from expressed sequence tags (ESTs). SNPs derived from EST are excellent markers for genetic mapping because of their representation of functional genes and potential for high throughput genotyping. SNPs can be developed by re-sequencing or by mining existing sequence databases. The public EST databases represent an enormous but heterogeneous repository of sequences, including many from a broad selection of oyster species and varieties. The significant redundancy within large EST collections makes them an attractive resource for rapid identification of SNPs. At the

present time, only a limited number of SNPs are available in the eastern oyster (*Crassostrea virginica*). In this study, we used a bioinformatics strategy to discover SNPs within an eastern oyster EST database. A collection of >9,000 eastern oyster ESTs was downloaded from GenBank and assembled into 602 contiguous sequences (contigs). The contigs were then visually inspected to identify SNPs with high minor allele frequencies and good flanking sequences. A total of 206 candidate SNPs from 100 genes were identified and characterized. Polymorphism information content for the SNPs ranged from 0.11 to 0.50. They are being validated and mapped in the eastern oyster.

**EFFECTS OF THE DINOFLAGELLATE *KARENIA BREVIS* ON LARVAL DEVELOPMENT IN THREE SPECIES OF BIVALVE MOLLUSC FROM FLORIDA.** Jay R. Leverone<sup>1</sup>, Norman J. Blake<sup>2</sup> and Sandra E. Shumway<sup>3</sup>. <sup>1</sup>Mote Marine Laboratory; University of South Florida, <sup>2</sup>University of South Florida, <sup>3</sup>University of Connecticut, Groton, CT.

The effects of *Karenia brevis* (Wilson clone) on larval survival and development of the bay scallop, *Argopecten irradians*, northern quahog, *Mercentaria mercenaria*, and eastern oyster, *Crassostrea virginica*, were studied in the laboratory. Larvae were exposed to cultures of whole and lysed cells, with mean total brevetoxin concentrations of 53.8 and 68.9  $\mu\text{g} \cdot \text{L}^{-1}$ , respectively. Survival of early (3-day-old) larvae was generally over 85% for all shellfish species at *K. brevis* densities of 100 cells  $\cdot \text{mL}^{-1}$  or less, and not significantly different between whole and lysed culture. At 1,000 cells  $\cdot \text{mL}^{-1}$ , survival was significantly less in lysed culture than whole culture for both *M. mercenaria* and *C. virginica*. Survival of late (7-day-old) larvae in all three species was not significantly affected by *K. brevis* densities of 1,000 cells  $\cdot \text{mL}^{-1}$  or less. At 5,000 cells  $\cdot \text{mL}^{-1}$ , however, survival was reduced to 37, 26 and 19% for *A. irradians*, *M. mercenaria* and *C. virginica*, respectively. Development of *C. virginica* and *M. mercenaria* larvae was protracted at *K. brevis* densities of 1,000 cells  $\cdot \text{mL}^{-1}$  and greater. These results suggest that blooms of *K. brevis*, and particularly their associated brevetoxins, may have detrimental consequences for Florida's shellfisheries by disrupting critical larval processes. Special attention should be paid to blooms of *K. brevis* where these shellfish naturally occur or where aquaculture and restoration activities are either ongoing or planned.

**RESPONSE OF BAY SCALLOP POPULATIONS TO LARVAL RELEASES AND AN UPDATE ON CURRENT RESTORATION ACTIVITIES IN PINE ISLAND SOUND, FL.** Jay R. Leverone<sup>1</sup>, Stephen P. Geiger<sup>2</sup>, William S. Arnold<sup>2</sup> and Jaime M. Greenawalt<sup>3</sup>. <sup>1</sup>Mote Marine Laboratory, University of South Florida, <sup>2</sup>Florida Fish and Wildlife Research Institute, <sup>3</sup>Sanibel-Captiva Conservation Foundation.

In 2003, we undertook a novel restoration approach to enhancing bay scallop populations in Pine Island Sound, Florida. This approach involved releasing competent, late-stage larvae into con-

tainment booms, which isolated the water column, thereby preventing the larvae from excessive dispersion. Three booms were used for larval releases and an additional boom served as a control (no larvae added). Larval settlement and juvenile recruitment were monitored throughout the following year. Scallop spat recruited to artificial substrates in all three treatments. Juvenile scallops, surveyed in Feb 2004, were found at all treatment locations while being absent from the control. Adult scallops, surveyed in July 2004, were two orders of magnitude greater at the restoration site than the resident scallop population within Pine Island Sound. Adult scallops were again surveyed in June 2005. That year, the entire survey area experienced a 100-fold increase in bay scallops from the previous year. Pine Island Sound had the highest abundance of bay scallops than any other Florida estuary in 2005. These results demonstrate that controlled release of competent larvae is a viable method for ultimately restoring bay scallop populations in Pine Island Sound. This fall, our restoration efforts expanded to South Pine Island Sound, a region that has also experienced reduced scallop densities over the past several decades. We will discuss some of the potential problems associated with restoration success at this location, including freshwater inputs and reduced salinity. We are also conducting or planning larval releases in three other Florida Gulf Coast estuaries (Sarasota Bay, Boca Ciega Bay and St. Andrews Bay) during 2005–2006.

**CHARACTERIZATION OF NRAMP IN *PERKINSUS MARINUS* AND *CRASSOSTREA VIRGINICA*.** Zhouer Lin<sup>1</sup>, José A. F. Robledo<sup>1</sup>, Gwendolyn Mullen<sup>2</sup> and Gerardo R. Vasta<sup>1</sup>. <sup>1</sup>COMB, UMBI, University of Maryland, Baltimore, MD 21202, USA, <sup>2</sup>Morgan State University Graduate School Science Education.

Nramp (natural resistance-associated macrophage protein) is a divalent cation transporter demonstrated to be a determinant of resistance/susceptibility to intracellular pathogens by preventing pathogens from acquiring divalent metals. Reciprocally, most parasites have developed efficient mechanisms for metal acquisition from their hosts. After characterizing Nramp in the oyster parasite, *Perkinsus marinus* (*PmNramp1*), we identified two additional Nramp species in our ongoing *Perkinsus* genome project (<http://www.tigr.org/tdb/e2k1/pmg/>, Microbial Genome Initiative, NSF). Neither intron boundaries nor intron numbers appear to be conserved: 7, at least 4, and 13 introns in *PmNramp1*, *PmNramp2*, and *PmNramp3*, respectively. *PmNramp1* and *PmNramp2* are more similar between them than with respect to *PmNramp3*, so far *PmNramp1* and *PmNramp3* appear to have a TATA-less promoter and the three *PmNramp* are expressed in *P. marinus* cultured trophozoites. We have found several polymorphic sites in *PmNramp1* and we are assessing their potential contribution(s) as virulence factors in the protozoan-oyster interaction. We are also characterizing *Crassostrea virginica* Nramp (*CvNramp*); *CvNramp* messages were identified in samples from hemocytes, mantle, gill,

muscle, and heart. Functional and immunolocalization studies of PmNramp and CvNramp are underway (Supported by Grant ICB-0321417, NSF).

**HYBRIDIZATION BETWEEN TWO GENERA OF CLAMS, *SPISULA SOLIDISSIMA* AND *MULINIA LATERALIS*, AND EARLY GROWTH TRIALS.** Scott Lindell, Bethany Walton, Janice Simmons and Steven Roberts, Marine Biological Laboratory, Woods Hole, MA.

The Atlantic surfclam (*Spisula solidissima*) and the coot clam (*Mulinia lateralis*) occupy different habitat niches along the eastern North American seaboard. The Atlantic surfclam supports a multi-million dollar fishery and has been the subject of pilot-scale commercial aquaculture. Among the interesting properties of the coot clam that have made it useful to biologists are its ease of culture, short generation time, and high reproductive rate. Despite their apparent geographic and reproductive isolation we have cross-bred these two species in the lab. Larvae of coot clams and both hybrids (*Spisula* eggs  $\times$  *Mulinia* sperm and *Mulinia* eggs  $\times$  *Spisula* sperm) metamorphosed at 10 days at 20°C and were then reared on set screens and in sand substrate. Results of the first four months of growth of the hybrids and *M. lateralis* will be presented here. We also present information about genetic markers for distinguishing hybrids from their parents. The results have implications for reassessing the taxonomy of macrclid clam species, and may hold promise for a new aquaculture candidate.

**ECONOMIC CONSIDERATIONS REGARDING THE RESTORATION OF CHESAPEAKE BAY OYSTER POPULATIONS USING NATIVE OR NON-NATIVE SPECIES.** Douglas Lipton, Tom Murray, and James Kirkley

The stated goal of the Environmental Impact Statement related to *Crassostrea ariakensis* introduction in the Chesapeake Bay is to restore the population to a level that could sustain a fishery equivalent to average landings from 1920–1970. Given the changing market for oysters on both the supply and demand side, it is unlikely that a fishery of this size, about 4.9 million bushels per year, is economically feasible. We suggest that a fishery is more likely sustainable at about 2.6 million bushels. In addition to the commercial oyster fishery there are a variety of other costs and benefits related to a restored oyster population. The benefits of a restored oyster population, given current knowledge, appear to be similar whether they are based on the native *C. virginica* or the introduced *C. ariakensis*. Thus, the major differences between the competing options of allowing or not allowing an introduction of a reproducing population of *C. ariakensis* appears to be a risk-time trade-off. The paper discusses the components of this risk-time trade-off in terms of market and non-market benefits and costs.

**DOES *PERKINSUS MARINUS* SECRETE EICOSANOIDS?**

Eric D. Lund, Fu-Lin E. Chu and Ellen Harvey, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

*Perkinsus marinus*, a protozoan parasite of the Eastern oyster, *Crassostrea virginica*, causes high mortalities in its host along the Atlantic and Gulf coasts of North America. Several virulence factors of *P. marinus* have been identified, but the mechanisms whereby *P. marinus* overwhelms the host immune response remain poorly understood. One class of virulence factors that has been identified in several protozoan parasites of mammals is eicosanoids, oxygenated metabolites of fatty acids. Previously we have demonstrated that *P. marinus* synthesizes a wide array of saturated, monounsaturated and polyunsaturated fatty acids, including arachidonic acid, a precursor to several eicosanoids including prostaglandins D<sub>2</sub>, E<sub>2</sub> and F<sub>2 $\alpha$</sub> . To determine if *P. marinus* produces eicosanoids in vitro meronts were cultured in a defined, lipid-free medium supplemented with cholesterol and screened for eicosanoids. GC/MS analysis of a derivatized extract of  $4 \times 10^8$  *P. marinus* meront cells revealed a peak with a spectrum similar to that of derivatized prostaglandin D<sub>2</sub> (PG D<sub>2</sub>), but no other derivatized eicosanoids were detected. Subsequent enzyme immunoassay (EIA) analyses of medium from *P. marinus* meront cultures ( $5\text{--}7 \times 10^5$  cells/ml) confirmed the presence of PG D<sub>2</sub> at concentrations between 40 and 140 pg/ml. These levels are comparable to those reported in the literature for *Trypanosoma* and *Plasmodium* species. The function of PG D<sub>2</sub> secreted by *P. marinus* is not currently known. However, prostaglandins synthesized by protozoan parasites of mammals have been shown to be immunosuppressive. This study was supported by a grant from the Metabolic Biochemistry, MCB Division (MCB-031108), National Science Foundation.

**ENERGY AND PROTEIN REQUIREMENTS FOR MAINTENANCE AND GROWTH IN THE SEA URCHIN (*PARACENTROTUS LIVIDUS*).** Ingrid Lupatsch, Esther Golombek and Muki Shpigiel, National Center for Mariculture Eilat Israel.

A novel approach to determining the protein and energy requirements in sea urchins is described here quantifying the requirements for energy and protein as the sum of the daily requirements for maintenance and growth. Two growth trials of each 91 and 95 days were performed at average temperatures of 22°C using sea urchins of 6.8 g and 12.4 g, respectively. The digestibility of the formulated diet was determined beforehand to contain 190 mg digestible protein and 14.4 kJ digestible energy per g feed. The requirement of digestible energy and protein for maintenance and growth were determined by feeding *Paracentrotus lividus* increasing levels, starting at zero and going up to apparent maximum feed intake. Total energy and protein gain in the urchins were measured by comparative slaughter technique. Protein content of whole live animals ranged from 3.7% initially up to 6.0% at the maximum feeding level corresponding to a gonadosomatic index (wet/wet) of

3.0 to 25.0 respectively. The relationship between digestible energy intake and energy gain was linear and the resulting slope describes the partial efficiency by the sea urchin for energy deposition. The digestible energy requirement for maintenance was calculated to be 32.8 J/g urchin / day and for digestible protein 0.56 mg/g urchin /day. The partial efficiency of utilization for growth was 0.25 and 0.39 for digestible energy and digestible protein respectively. Using these values together with a suitable growth prediction allow optimization of feeds to contain the proper energy to protein ratio for *P. lividus*.

**QUANTIFICATION OF QUAHOG PARASITE UNKNOWN (QPX) IN ENVIRONMENTAL SAMPLES.** M. Maille Lyons<sup>1</sup>, Steven Roberts<sup>2</sup>, Chris Dungan<sup>3</sup>, Roxanna Smolowitz<sup>2</sup> and J. Evan Ward<sup>1</sup>. <sup>1</sup>University of Connecticut, 1080 Shennecossett Ave, Groton, CT, <sup>2</sup>Marine Biological Laboratory, Woods Hole, MA, <sup>3</sup>Maryland Department of Natural Resources.

Quahog Parasite X (QPX) is a pathogen of the bivalve mollusc, *Mercenaria mercenaria*, (northern quahog or hard clam). The objective of this study was to develop and validate a quantitative polymerase chain reaction (qPCR) assay that is sensitive and specific for detection of the QPX parasite in experimental and environmental samples. Unique aspects of this qPCR assay development include quantifying the serial dilutions of QPX cells before DNA extraction to calibrate standard curves to cell counts and the targeting of a novel potassium channel gene for amplification. Sensitivity was evaluated with serial dilutions of cultured cells to determine the lowest concentration of DNA that remained detectable in the presence and absence of potential inhibitory environmental compounds. Based on our results, the qPCR assay can effectively quantify QPX within the range of one to several thousand organisms. Specificity was assessed by testing 29 thraustochytrid-like protist strains isolated from oysters (*Crassostrea virginica*, *Crassostrea ariakensis*), softshell clams (*Mya arenaria*) or quahogs (*M. mercenaria*) from Virginia, Chesapeake Bay, China, or Oregon. This technique will provide a valuable tool for characterizing QPX abundances in coastal environments and for improving QPX disease diagnoses in clams.

**EVALUATION OF ARTIFICIAL REEF DESIGN FOR OYSTER HABITAT RESTORATION IN GEORGIA.** Justin Manley<sup>1</sup>, Randal L. Walker<sup>1</sup>, Alan Power<sup>1</sup>, Dorset Hurley<sup>2</sup>, Mathew Gilligan<sup>3</sup> and Joseph P. Richardson<sup>3</sup>. <sup>1</sup>University of Georgia Marine Extension Service, 20 Ocean Science Circle, Savannah, GA 31405, <sup>2</sup>Sapelo Island National Estuarine Research Reserve, <sup>3</sup>Savannah State University.

The eastern oyster, *Crassostrea virginica*, is a common estuarine species along the eastern seaboard of the United States and forms dense intertidal fringing reefs in coastal Georgia. Oyster reef acreage has decreased in Georgia over the last century as a result

of commercial harvesting, rapid coastal development, and disease epidemics (*Perkinsus marinus* and *Haplosporidium nelsoni*). Spat abundance is not the limiting factor (settlement rates up to 204,700 m<sup>2</sup>); rather it is the availability of cultch. Southeastern estuaries are dominated by muddy substrates with oyster reefs forming the only hard substrate. The insufficient return of shell has been detrimental to sustaining oyster populations. The purpose of this study was to determine an optimal cultch material and design for oyster restoration in this environment. Fresh oyster shell, old washed oyster shell, and whelk shell were separately placed into crab traps and plastic mesh bags. Spat collection sticks (PVC tubes embedded with calcium carbonate chips) were arranged in densities of 81 m<sup>2</sup> and 25 m<sup>2</sup>. Treatments were placed in the Duplin River, Sapelo Island in April 2004. Oyster reef acreage in this area decreased from 8.9 ha in 1891 to 4.3 ha by 2000. Mean annual oyster growth and mortality rates were assessed for each treatment after a one-year period. The collection sticks in the 81 m<sup>2</sup> treatment performed best (mean annual growth rate: 77.09 mm/year, mortality: 3.467 ± 1.423 %). Structures with higher vertical placement reduce complications associated with high siltation rates and may provide greater surface area for larval attachment.

**AGE-SPECIFIC MORTALITY RATES AND CAUSES OF MORTALITY IN NATURAL OYSTER, *CRASSOSTREA VIRGINICA*, POPULATIONS.** Roger Mann, Melissa Southworth, Juliana M. Harding and Ryan Carnegie. Virginia Institute of Marine Science.

There are very few estimates of natural mortality rates in natural populations of oysters, *Crassostrea virginica*, (as opposed to abundant estimates from culture and tray type experiments). We offer two methods to estimate natural mortality rates over extended periods and multiple reefs systems for populations in the James River, VA under varying disease challenges. We convert size specific live and box data to age specific demographics, and then generate estimates of age specific mortality from either box ratio (#box / (#box + #live)) or inverse survivor ratios (Live(t) – Live(t + 1)/Live(t)). These are presented and compared with respect to oyster population density, prevalence and intensity data for *Perkinsus marinus*, and river flow data as a surrogate for salinity.

**YOU CANNOT GET THERE FROM HERE, CAN YOU? AN EXAMINATION OF THE TIME COURSE AND IMPACT OF OYSTER RESTORATION IN CHESAPEAKE BAY.** Roger Mann, Juliana M. Harding and Melissa Southworth. Virginia Institute of Marine Science.

While restoration of the oyster resource of the Chesapeake Bay is supported by extensive research and "on the ground" efforts there has been a reluctance to estimate a realistic time course for achieving both restoration goals and eventual magnitude of impacts on ecological processes, water quality and support of com-

mercial fisheries (noting that these may not be equivalent). Fundamental aspects of oyster biology dictate that such estimates will have similar limitations no matter what species (singular or plural, native or non-native) is considered. We describe the current state of knowledge of stock-recruit relationships, annual replacement ratios, growth rates, age-specific mortality rates in relation to density and disease pressure, and available habitat to provide upper and lower estimates of time to stable terminal carrying capacity (with implications for ecological services and water quality) and potential harvest for commercial purposes.

**CONDITION INDICES OF THE EASTERN OYSTER AND REEF CHARACTERIZATION AS RELATED TO WATER QUALITY IN THREE TIDAL CREEKS.** Anne L. Markwith, Martin H. Posey and Troy D. Alphin. UNC-Wilmington. Center for Marine Science, 5600 Marvin K. Moss Lane, Wilmington, NC 28409.

The eastern oyster (*Crassostrea virginica*) is an ecologically and commercially important species, providing a series of ecosystem functions (erosion control, filtration, habitat nutrient cycling). This study focuses on the dynamic interaction between oysters and water quality. Through filtration and increased sedimentation oysters have been shown to reduce particulate load in the water column. However, oyster condition and health are also closely linked to water quality conditions, especially total suspended solids (TSS) and sedimentation rates. We expect to see a correlation between the general background water quality, general oyster reef characteristics (density, percent cover, size demography, and rugosity) and measures of oyster condition, such as soft tissue dry weight, volume, and shell characteristics. Data was collected during two sampling periods in three tidal creeks in southeast North Carolina that vary in background water quality characteristics. Sampling occurred during summer (July/August) 2005 and during winter (December 2005/January 2006). All sampling was conducted in the middle section of the creeks, where tidal regime and salinity were similar among sites. Condition index was performed on fifteen randomly selected oysters in two different size ranges per creek using Hopkins' formula:  $C.I. = (\text{weight of dry tissue})(100)/(\text{internal shell volume})$ . Water quality data and information on watershed development was provided by a long term monitoring project. It was hypothesized that the healthier oysters would be found in the creeks with the historically better water quality levels. Preliminary analysis of the data from the creeks however does not support the hypothesis.

**CONDITION INDEX OF SURFCLAMS (*SPISULA SOLIDISSIMA*) IN THE MID-ATLANTIC BIGHT.** Rebecca J. Marzec, Yungkul Kim and Eric N. Powell. Haskin Shellfish Research Laboratory, Rutgers University.

Scientists from the Haskin Shellfish Research Laboratory and National Marine Fisheries Service-Northeast Fisheries Science Center conducted a survey of surf clam (*Spisula solidissima*)

stocks in an area from northern New Jersey to southern Virginia during June and July of 2004 in order to evaluate the progress of mortality, apparently related to warming of the Mid-Atlantic Bight. One component of the survey was the measurement of condition index at 104 locations from Delmarva to Long Island. The program followed a pilot study in 2002 that suggested that surf clam mortality off Delmarva was likely caused by warmer temperatures decreasing feeding and subsequently leading to starvation. All sampling was conducted aboard the *F/V Lisa Kim*, a commercial clammer. Condition index was highest inshore, with the exception of a few of the most inshore stations, and lowest at the offshore edge of the clam's range. An estimate of meat weight for a standard 120-mm clam, from site-specific length-weight regressions, revealed that the animals near the center of the inshore-offshore distribution had a greater weight for a given length than those living at the edges of the clam's range, probably due to the influence of temperature on feeding and growth. Low condition in the extreme inshore locations suggests that warmer temperatures continue to negatively affect surf clam nutrition and indicate the continued susceptibility of clams along the southern and inshore boundary to warming in the Mid-Atlantic Bight.

**FOULING CONTROL FOR OFF-BOTTOM OYSTER CULTURE USING THE ADJUSTABLE LONGLINE SYSTEM.** Vanessa Maxwell and John Supan. Louisiana Sea Grant Development Program, Sea Grant Bldg., Louisiana State University, Baton Rouge, LA 70803.

The Eastern oyster, *Crassostrea virginica*, is an important commercial species in Louisiana. The production of oysters from Louisiana has decreased due to predation, habitat destruction, disease, and the moratorium on leases. Off-bottom culture may be a feasible alternative to increase production, but fouling organisms, such as barnacles and bryozoans, can obstruct mesh openings, reducing growth and survival, and increasing maintenance. The Adjustable Longline System (ALS), commercially used in Australia, allows the oysters to be held at various depths to control culture conditions. This study used the ALS to determine a method of controlling biofouling by measuring 1) growth, 2) survival, and 3) fouling. At the Sea Grant Grand Isle Oyster Hatchery, the ALS was used to test 4 different treatments: a) weekly aerial drying; b) a monthly brine dip; c) daily exposure at low tide; and, d) a no treatment control. Shell height was measured monthly. Fouling was measured monthly by counting organisms on ceramic tiles held on longline cable alongside oyster bags. Total fouling on the oysters was measured by volume at harvest. Oysters reached the legal market size of 76 mm using the ALS within 12 months. Weekly aerial drying had less fouling than either the control or brine dip treatments. The low-tide treatment had the least fouling, but the slowest growth rate. The efficient treatment was weekly aerial drying.

**DERMO DISEASE IN STANDARD AND DISEASE-TOLERANT STRAINS OF THE OYSTER (*CRASSOSTREA VIRGINICA*) IN THE PATUXENT RIVER, MD.** Carol B. McCollough<sup>1</sup>, Christopher F. Dungan<sup>1</sup> and George R. Abbe<sup>2</sup>.

<sup>1</sup>Maryland DNR, Fisheries Service, Cooperative Oxford Laboratory, 904 S. Morris Street, Oxford, MD 21654, <sup>2</sup>Morgan State University, St. Leonard, MD 20685.

Disease-tolerant strains of the eastern oyster may be of value in population restoration efforts if they outperform the standard strain in survival and growth. Dermo disease measures in two disease-tolerant strains, DEBY and CROSBreed, were compared to those in standard oysters deployed along a salinity gradient in the Patuxent River. In September 2003 tolerant strains were deployed at a size of 3–4 mm; standards were deployed at 16 mm. Whole body burden dermo disease assays were conducted monthly from the time of deployment. Salinities were depressed in 2003 due to above average freshwater inflows. During 2004 and 2005, flows in the Patuxent River returned to normal ranges. *Perkinsus* sp. infections were detected in all strains at all sites during 2004. Typical seasonal patterns in prevalence developed during both 2004 and 2005. Mean sample body burdens from all strains rarely exceeded 30 parasites/gm tissue wet weight, and were typically less than one, except at the downriver site in September 2005 where body burdens in standards and DEBYs reached and exceeded 10<sup>2</sup> parasites/g. DEBYs exhibited delayed or depressed prevalence peaks compared to CROSBreeds and standards at all sites during the second year; however, prevalences in this strain were not consistently lower than both the others, except at the mid-river site. At this time, strong differences in disease progression between strains are not apparent, but may develop during the third year as these experimental oysters endure further disease pressure as they grow to market size.

**RESEARCH PROGRESS ON WHITE ABALONE (*HALIOTIS SORENSENI*).** Thomas B. McCormick, Channel Islands Marine Resource Institute.

White abalone (*Haliotis sorenseni*), a large gastropod mollusc in the family *Haliotidae*, was once found in rocky subtidal ocean waters off the west coast of North America from Point Conception in California, south to Punta Abreojos, Baja California, Mexico. Long recruitment intervals, site fidelity, fishing, poaching, disease, and habitat change have contributed to its decline. Surveys of historic habitat in the 1990s indicated that population densities had dropped to less than 0.1% of the estimated pre-exploitation population size. A status review led to a 2001 listing of the white abalone as the first endangered marine invertebrate under the Endangered Species Act of 1976 (66 FR 29053). A cooperative program of research is now providing information many aspects of white abalone biology. Large-scale cultivation of hatchery-raised

white abalone is providing an opportunity to study all life stages of this invertebrate. This presentation will provide a summary of research on age at first sexual maturation, optimal cultivation temperature, density, and diet. Behavioral studies suggest that white abalone are capable of rafting on macroalgae, and may have far greater dispersal capabilities than previously thought.

**USE OF A BIOENERGETICS MODEL TO INVESTIGATE THE HABITAT USE AND BEHAVIOR OF A NONINDIGENOUS CRAB, *CARCINUS MAENAS*.** P. Sean McDonald<sup>1</sup>, Kirstin K. Holsman<sup>1</sup>, David A. Beauchamp<sup>1</sup>, Brett R. Dumbauld<sup>2</sup> and David A. Armstrong<sup>1</sup>. <sup>1</sup>University of Washington, SAFS, <sup>2</sup>USDA, Agricultural Research Service.

We developed an individual-based bioenergetics model to investigate habitat use and migration behavior of non-indigenous European green crab (*Carcinus maenas*) in Willapa Bay, Washington, USA. The model was parameterized using existing studies and applied to four scenarios of habitat use and behavior during a 214-day simulation period (April–October), including crabs occupying (1) sublittoral habitat, (2) sublittoral habitat but undertaking intertidal migrations (3) mid-littoral habitat, and (4) high littoral habitat. Monthly trapping was done along an intertidal gradient to determine the distribution of crabs for the same time interval as the simulation period, and we compared model results to the observed pattern. Model estimates suggest no intrinsic energetic incentive for crabs to occupy littoral habitats since metabolic costs were ~6% higher for these individuals than their sublittoral counterparts. Crabs in the littoral simulations were also less efficient at converting consumed energy into growth than sublittoral crabs, yet trapping revealed abundant *C. maenas* in mid-littoral habitats of Willapa Bay and no evidence of resident sublittoral populations. The discrepancy intimates the significance of interspecific interactions that are not incorporated into the model but nonetheless increase metabolic demand. Agonistic encounters with native Dungeness crabs (*Cancer magister*) may be chief among these added costs, and *C. maenas* may largely avoid interactions by remaining in littoral habitats neglected by native crabs. We suggest that *C. maenas* in Willapa Bay occupy tidal elevations that minimize encounters, and thus metabolic costs, while simultaneously maximizing submersion time and foraging opportunities.

**DISTRIBUTION AND QUANTIFICATION OF MULTIXENOBIOTIC RESISTANCE PROTEINS IN THE SEA URCHIN, *LYTECHINUS ANAMESUS*.** Nature A. McGinn and Gary N. Cherr. University of California-Davis, Bodega Marine Laboratory, P.O. Box 247, Bodega Bay, CA 94923.

Multixenobiotic resistance (MXR) is an efflux mechanism that uses transport proteins to remove foreign chemicals and waste products from cells. A taxonomically diverse group of marine



organisms express MXR transport proteins and in many larval forms, MXR appears to be a first line of defense against natural toxins and contaminants. Research has shown that marine species express transport proteins similar to mammalian P-glycoprotein (P-gp) and multi-drug resistance-associated proteins (MRPs). MXR proteins are found in molluscan gills where MXR also appears to play a protective role. However, few studies have examined other adult tissues in marine species for expression of MXR proteins. In mammals, homologous multidrug resistance (MDR) proteins occur in tissues with important protective functions including the blood brain barrier and placenta. In this study, we are using the sea urchin, *Lytechinus anamesus*, to investigate the expression and potential function of MXR transport proteins in an adult model marine organism. Preliminary Western blot analysis with a monoclonal antibody indicates expression of P-gp in adult sea urchin tissues including gonads and coelomocytes. We are surveying adult tissues including gonads, gut, tube feet, and coelomocytes as well as major developmental stages for expression of P-gp and MRPs. Study of the larval stages will help elucidate potential transitions in expression and function of the MXR proteins from developing to adult sea urchins. Results from Western blot analysis and immunohistochemistry with custom sea urchin antibodies will be presented. Protein expression data can serve as a baseline for future toxicological research with sea urchins.

**THE EFFECTS OF AMMONIA ON THE DEVELOPMENT, SURVIVAL AND METAMORPHIC SUCCESS OF *STROMBUS GIGAS* VELIGERS.** Melissa McIntyre<sup>1</sup>, Megan Davis<sup>2</sup> and Amber Shaw<sup>1</sup>. <sup>1</sup>Florida Institute of Technology, Melbourne, FL. <sup>2</sup>Harbor Branch Oceanographic Institution, Ft. Pierce, FL.

The Florida Keys queen conch population was a vital fishery, but heavy fishing pressure forced the closure of the commercial fishery in 1976. Since the closure, the recovery of the population has been minimal due to low recruitment of veligers. The queen conch larval stage lasts two to six weeks and during this time veliger survival may be affected by contaminants. Ammonia enters the water through sewage leaching and agricultural runoff. Ammonia is toxic to coral planulae, but toxicity of  $\text{NH}_3$  to queen conch larvae has not been established. This study examined the effects of  $\text{NH}_3$  on new hatch, five days old, 10 days old, and competent veligers. The treatments tested were artificial seawater; natural seawater; and 1, 10, 100 and 1000  $\mu\text{M}$  of ammonia. Development, mortality and an  $\text{LC}_{50}$  was determined for each larval stage. Newly hatched veligers exposed to ammonia had a 15–30% increase in mortality over the control after 96 hours. Five day old veligers had 100% mortality in the  $\text{NH}_3$  treatments at 96 hours and an  $\text{LC}_{50}$  of 0.8  $\mu\text{M}$ . Ten day old and competent veligers were robust and able to withstand exposure to levels below 1000  $\mu\text{M}$ .

with  $\text{LC}_{50}$ 's of 429 and 576  $\mu\text{M}$ , respectively. Metamorphosis was observed in all treatments, excluding 1000  $\mu\text{M}$ ; with natural seawater having the greatest metamorphic success. This study reveals that exposure to  $\text{NH}_3$  can compromise the development, survival, and metamorphic success of *Strombus gigas*. The findings of this study will assist in understanding the role ammonia has on recruitment of veligers in the Florida Keys.

**COMPARISON OF *CRASSOSTREA ARIAKENSIS* AND *C. VIRGINICA* IN THE DISCHARGE AREA OF A NUCLEAR POWER PLANT IN CENTRAL CHESAPEAKE BAY.** Richard I. McLean<sup>1</sup> and George R. Abbe<sup>2</sup>. <sup>1</sup>Maryland Department of Natural Resources. <sup>2</sup>Morgan State University.

The potential for introduction of the non-native *Crassostrea ariakensis* into Chesapeake Bay prompted a five month comparison study with *C. virginica* conducted near the Calvert Cliffs Nuclear Power Plant in Maryland, with the primary goal being a comparison of uptake rates of radionuclides released by the plant. The study began with approximately 200 market size triploid *C. ariakensis* (1-yr old) and 200 diploid *C. virginica* (3-yr old) held in secure stainless steel cages 0.5 m off bottom. Half were deployed from July to September 2004, and half from July to December. Although quantities of radionuclides released by the CCNPP were insufficient to yield detectable concentrations in either species, other data were obtained. From July to December, *C. ariakensis* increased its initial shell length by nearly 20% compared to 9% for *C. virginica*. Although mean shell sizes of both species were similar, meat weights and condition indices for *C. ariakensis* were significantly greater ( $p < 0.001$ ) than for *C. virginica*. *C. ariakensis* had lower overall mortality even though nine were lost to blue crab predation during the first exposure period. After five months of exposure, prevalence of Dermo disease in *C. virginica* was 65% with a relative intensity of 1.50 on a 0–7 scale; *C. ariakensis* showed no sign of infection. Although the non-native *C. ariakensis* may have much to offer with high meat yield and disease resistance, rapid growth of triploids results in very thin shells, which may make them more susceptible to blue crab predation than the thicker-shelled *C. virginica*.

**GENOMIC ANALYSIS OF GROWTH IN LARVAE OF *CRASSOSTREA GIGAS*.** Eli Meyer, Dennis Hedgecock and Donal T. Manahan. University of Southern California, Los Angeles, CA.

Molecular biological analysis has provided important insights into the processes of animal development. However, data on the molecular biological bases of growth are more scant. In particular, the molecular biological bases of hybrid vigor have long remained unresolved despite nearly a century of research and enormous



economic importance. We approached this question by comparing global gene expression patterns in slow- and fast-growing larval families of the Pacific oyster, *Crassostrea gigas*. These different families of larvae were produced through factorial crosses between inbred lines. A total of 4.5 million cDNAs were cloned from 6-day-old larvae and analyzed by "Massively Parallel Signature Sequencing" (Solexa, Inc.). This analysis resulted in quantitative expression profiles and partial sequence data for each of 52,825 unique cDNAs present. Of these, a small subset of 188 genes was found to be differentially expressed in fast-growing hybrid larvae. By comparison with GenBank, 51 of these cloned genes showed significant similarity with DNA sequences for which a function is known; 23 of which represent ribosomal proteins. We found that 40% of these ribosomal proteins were up-regulated and the other 60% down-regulated in fast-growing larvae. These data demonstrate the feasibility of functional genomic approaches and reveal that regulation of protein synthesis machinery is involved in the molecular mechanisms responsible for hybrid vigor. This approach has resulted in the generation of a set of gene probes and possible "markers" that will likely prove useful as early predictors of rapid growth potential in oysters.

**THE USE OF MOLECULAR MARKERS TO ASSESS NATIVE OYSTER RESTORATION EFFORTS.** Coren A. Milbury and Patrick M. Gaffney, University of Delaware, College of Marine Studies, Lewes, DE 19958.

Restoration efforts are becoming essential in managing many of our ecological resources. Monitoring the efficacy of these programs is essential for evaluating their cost-effectiveness and testing alternative restoration techniques. A majority of oyster restoration efforts use hatchery-produced strains derived from a native stock; however, identifying seed derived from the native population is difficult and requires a high degree of specificity and accuracy. Recent advances in genetic techniques provide high-throughput cost-effective methods for discriminating outplanted seed and their progeny from natives. Our approach is based on shifts in mitochondrial haplotype frequencies occurring during the course of hatchery propagation. Typical natural populations of oysters possess a single major haplotype and an abundance of rare haplotypes. As a result of population bottlenecks during propagation, hatchery seed will often exhibit elevated frequencies of one or more haplotypes that are rare in the source population, allowing discrimination between hatchery seed and natives. Using high-throughput screening techniques, we have identified mitochondrial single nucleotide polymorphisms (mtSNPs) that distinguish hatchery seed planted in the Chesapeake Bay. These markers will be used to determine if outplanted oysters survived and contributed to local recruitment. Even when enhancement stocks are derived from local broodstocks, high-throughput molecular genotyping techniques can allow effective monitoring of enhancement efforts.

**DIGESTIVE ENZYME ACTIVITY OVER ONTOGENY IN BAY SCALLOPS, *ARGOPECTEN IRRADIANS*, AND SEA SCALLOPS, *PLACOPECTEN MAGELLANICUS*.** Lisa M. Milke, V. Monica Bricelj and Neil W. Ross, National Research Council, Institute for Marine Biosciences, 1411 Oxford St., Halifax, NS, B3H 321 Canada.

Although stage- and species-specific differences in dietary requirements have been demonstrated for bivalves, the mechanisms responsible for differential diet performance remain largely unexplained. While biochemical composition is central to assessing the nutritional value of a diet, an animal's digestive and assimilation capacity are equally important. Digestive capacity of sea and bay scallops may differ 1) over ontogeny associated with morphological changes, and 2) between species, resulting from adaptation to different environments and food supply. To address this, scallop postlarvae [initial shell height (SH) ~250 µm] were reared on a mixed algal diet consisting of one diatom and one flagellate until 4–5 mm SH, and were sampled at intervals coinciding with major morphological changes. Colorimetric assays were conducted to measure the specific activity of carbohydrases (laminarinase, cellulase and alpha-amylase), proteases (azocasein and cathepsin B) and lipase (esterase) in scallop tissues. While preliminary results show no pattern in protease or lipase activity either between species or over development, clear differences in carbohydrase activities were observed. Sea scallops exhibited higher laminarinase activity, but lower levels of alpha-amylase and cellulase than bay scallops, with the most pronounced changes in enzymatic activity occurring prior to attaining ~1.2 mm SH. However, laminarinase activity was significantly higher than activities of either alpha-amylase or cellulase in both scallop species. Our findings have implications for developing targeted diets for commercial hatcheries and increasing our understanding of bivalve-seston interactions in the field.

**NATIVE FLORIDA CRUSTACEAN PREDATORS' PREFERENCES REGARDING THE NON-INDIGENOUS GREEN MUSSEL, *PERNA VIRIDIS*.** Emily Mitchem<sup>1</sup>, Jonathan S. Fajans<sup>2</sup> and Shirley M. Baker<sup>1</sup>. <sup>1</sup>University of Florida, <sup>2</sup>Florida Institute of Oceanography.

The green mussel, *Perna viridis*, a native of the Indo-Pacific, was first reported in Tampa Bay in 1999 (Benson et al. 2001; Ingrao et al. 2001). In order to gain a better view of what this invasion might mean for Florida's waters and its native species, this experiment posed the following two-fold question: (1) Will native crustacean predators prey on *P. viridis*? and (2) Will these predators prefer native bivalves over *P. viridis*? Due to the high commercial value of *Panulirus argus*, the Caribbean spiny lobster, and *Callinectes sapidus*, the blue crab, these crustaceans made suitable predator subjects. The eastern oyster, *Crassostrea virginica*, and hard clam, *Mercenaria mercenaria*, were chosen as competing prey because these bivalves are commercially impor-

tant within Florida. Crustaceans and bivalves were placed in a recirculating system with eight 624 liter tanks. Filtration systems included a protein skimmer and fluidized bed, wet-dry filter, and ultraviolet sterilization. Trials ran for fourteen days. *P. argus*, consumed one *C. virginica*, and seventeen *M. mercenaria*. *C. sapidus*, consumed fifteen *P. viridis*, seven *C. virginica*, and seven *M. mercenaria*. A one-way analysis of variance was used to analyze data. A p-value less than 0.05 was considered statistically significant. For the *P. argus* experiment,  $p = 0.0219$ , showing that *P. argus* data were significant. After this, a Tukey Test was performed. *C. sapidus* data were not statistically significant because  $p = 0.2067$ , although *C. sapidus* consumed twice as many *P. viridis* than either *M. mercenaria* or *C. virginica*.

**MULTIPLE AXES OF VARIATION IN NATURAL POPULATIONS: A COLLISION OF MORPHOLOGY, GENETICS, AND TAXONOMY.** Karen E. Mock<sup>1</sup>, Jer Pin Chong<sup>1</sup>, Jayne Brim-Box<sup>2</sup> and Jeanette Howard<sup>2</sup>. <sup>1</sup>Utah State University, <sup>2</sup>Confederated Tribes of the Umatilla Indian Reservation.

Appropriate classification of taxa is essential for the effective research and management of natural populations. This task is difficult, however, because variation in natural populations occurs along several different, and often incongruent, axes. The difficulty is confounded by disagreement among taxonomists regarding appropriate species definitions. Using a recent example of genetic differentiation among *Anodonta* populations in the Columbia River basin, these issues will be explored as they apply to freshwater mussels.

**SABELLID POLYCHAETE INFESTATIONS OF FARMED CALIFORNIA ABALONE.** James D. Moore<sup>1</sup>, Christy I. Juhasz<sup>2</sup> and Thea T. Robbins<sup>1</sup>. <sup>1</sup>California Department of Fish and Game, P.O. Box 247, Bodega Bay, CA 94923, <sup>2</sup>UC Davis, Bodega Marine Laboratory.

The South African sabellid polychaete, *Terebrasabella heteromacinata*, lives in shells of abalone. Accidentally introduced to California during the 1980s, it was spread via abalone shipments to abalone culture and display facilities throughout the state. Eradication efforts have been largely successful although concern remains about whether it has become established in intertidal gastropod populations adjacent to previously-infested facilities. We found that sabellid transmission can occur among native turban snails *Tegula funebris*, corroborating the potential risk of sabellid infestations in native, non-haliotid gastropod populations. Nevertheless, a state-wide survey of turban snails and limpets at 21 exposed or potentially exposed locations found no sabellids, suggesting that the pest has not become established in California. Freshwater treatment is commonly used to sanitize holding units,

hands and equipment at farms and display facilities, but the amount of freshwater required to kill sabellids and their larvae was not well defined. We found that sabellid transmission occurred from infested abalone shells that had been immersed in freshwater for up to eight hours. Additionally, motile sabellid larvae survived up to 32 seconds of immersion in freshwater. We produced an informational video to provide facility staff with recommended practices to achieve eradication and minimize chances of reinfestation. Supported in part by the University of California Integrated Pest Management Program and the Marine Region, California Department of Fish and Game.

**COMPARISON OF BACTERIA UPTAKE AND DEPURATION BETWEEN *CRASSOSTREA ARIAKENSIS* AND *CRASSOSTREA VIRGINICA*.** James A. Morris Jr.<sup>1</sup>, Tanya J. Bean<sup>2</sup>, Rachel T. Noble<sup>3</sup> and Patricia K. Fowler<sup>2</sup>. <sup>1</sup>Center for Coastal Fisheries and Habitat Research, National Centers for Coastal Ocean Science, NOAA, Beaufort, NC 28516, <sup>2</sup>North Carolina Department of Environment and Natural Resources, Division of Environmental Health Shellfish Sanitation and Recreational Water Quality Section, Morehead City, NC 28557, <sup>3</sup>University of North Carolina, Institute of Marine Sciences, Morehead City, NC 28557.

Considerations to introduce the Suminoe, or Asian oyster, *Crassostrea ariakensis*, along the East Coast have raised many questions regarding ecology, economics, and human health. To date, research has focused primarily on the ecological and socioeconomic implications of this initiative, yet few studies have assessed its potential impact on public health. Our work compares the rates of bioaccumulation and depuration of indicator organisms (such as *E. coli*) and *Vibrio* sp. between *C. virginica* and *C. ariakensis* in the laboratory. Preliminary results suggest that the rates of bioaccumulation of *E. coli* in *C. ariakensis* were significantly lower than those for *C. virginica* and that depuration was variable between the two species. This research provides coastal managers with insight into *C. ariakensis* response to bacteria, an important consideration for determining appropriate management strategies for this species. Further field-based studies will be necessary to elucidate the mechanisms responsible for the differences in rates of bioaccumulation and depuration.

**SEANEST—A COMMERCIAL SYSTEM FOR LARGE SCALE CULTURE OF BENTHIC SPECIES.** Vidar Mortensen, Praktisk Teknologi AS, Norway.

Sea based culture of benthic invertebrates is very labour intensive. This is especially true for species like sea urchin, which require frequent feeding. In addition, traditional equipment like oyster trays fails to accommodate the biological demands of sea

urchins. The SeaNest system has been developed to solve these problems, and to provide the farmer with a modern workplace. Time consuming physical labour is avoided through automatic handling of the holding boxes, and the operator has an ergonomically good position while running the system. The system is designed to meet the biological requirements of sea urchins, with respect to surface area, water exchange, waste removal and short time of air exposure. Further developments towards automatic feeding, cleaning and tracking of holding boxes will provide additional benefits for the farmer. The systems main concepts and technical features will be presented. Prototypes of the system are in use at a commercial sea urchin farm in Norway. The experiences from this farm will be discussed both with regards to sea urchin farming in general, and the function of the SeaNest system in particular. (The SeaNest system is patented, with further patents pending).

**OBSERVATIONS OF ASIAN *PERKINSUS* SPECIES AND *PERKINSUS MARINUS* IN THE SUMINOE OYSTER, *CRASSOSTREA ARIAKENSIS*.** Jessica A. Moss and Kimberly S. Reece, VIMS, The College of William and Mary, P.O. Box 1346, Gloucester Point, VA 23062.

The decline of *Crassostrea virginica* populations has led to an interest in using the non-native oyster species, *Crassostrea ariakensis*, for aquaculture development, fishery resource enhancement and habitat restoration in the Chesapeake Bay region. Previous field studies comparing the performance of *C. ariakensis* and *C. virginica* in the region suggested that the non-native oyster was substantially more tolerant of the endemic protozoan parasite, *Perkinsus marinus*. We conducted a disease survey of *C. ariakensis* in China, Japan and Korea using molecular diagnostics. PCR-based assays revealed two *Perkinsus* species in Asian Suminoe oyster populations that currently are not found in US waters: *Perkinsus olseni* and, based on DNA sequence data, an undescribed *Perkinsus* species. We will report on progress towards molecular characterization of this undescribed *Perkinsus* species. Quarantine protocols for *C. ariakensis* brought to the US from Asia should limit introduction of exotic pathogens, however, it is important to understand the impact that Asian *C. ariakensis* pathogens could have on local bivalve species in case of accidental introduction via the host or through an indirect source such as ballast water, in which case the non-native oyster could act as a pathogen reservoir. We will report results of challenge experiments being conducted with Asian *C. ariakensis* pathogens to examine the impacts on the local Chesapeake Bay bivalve species, *C. virginica* and *Mercenaria mercenaria*, as well as on *C. ariakensis*. During these experiments, we unexpectedly observed the acquisition of moderate and heavy intensity infections of *P. marinus* in *C. ariakensis* oysters being maintained in the laboratory.

**GENOMIC EVIDENCE FOR DIVERGENT SELECTION IN EASTERN OYSTER POPULATIONS ACROSS AN ECOTONE.** Maria Murray and Matthew P. Hare, University of Maryland.

The eastern oyster, *Crassostrea virginica*, is broadly distributed along the western North Atlantic coastline. Inside this range, a steep latitudinal temperature gradient and a transition in species composition from temperate to subtropical communities generate an ecotone along eastern Florida. Despite evidence for homogenizing gene flow within large continental regions, presumably mediated by a three-week planktonic larval stage, oyster populations display a sharp genetic cline in eastern Florida. We are interested in discovering the abiotic and biotic mechanisms by which a high gene flow species maintains genetic diversity along the ecotone. Population genomics approaches offer effective ways to statistically test for genetic evidence of divergent selection in this non-model species. We report genomic evidence for the role of selection across the ecotone using 215 amplified fragment length polymorphism loci sampled from two populations experiencing the environmental extremes. Simulations of neutral population divergence provided a statistical framework for testing whether differentiation at some loci is greater than expected by genetic drift. For a small portion (2–7%) of loci, allele frequency differentiation was high enough across the ecotone to reject the neutral drift model ( $p < 0.01$ ). Corroborating this inference with additional population sampling, several of the nonneutral AFLP loci show clinal variation concordant with previously described loci. Also, one AFLP locus was converted to a codominant marker and still shows a significantly nonneutral pattern of differentiation. Thus, we infer that divergent selection along the Atlantic Florida ecotone helps maintain genetic differences between oyster populations in the Atlantic and Gulf of Mexico.

**EASTERN OYSTER AND HARD CLAM CULTURE FOR THE REDUCTION OF RAS EFFLUENT NUTRIENT COMPOSITION.** Amanda Myers, Wade Watanabe and Ami E. Wilbur, University of North Carolina, Wilmington, 7205 Wrightsville Ave., Wilmington, NC 28403.

Closed recirculating aquaculture systems (RAS) are a technological innovation that reduces the amount of water needed for culture by treating and reusing >90% of the total water volume daily. Such systems consequently produce substantially smaller volumes of effluent, but this smaller volume is substantially more enriched in nutrients than effluent from flow-through or open systems. For RAS to emerge as a viable culture strategy, an economical and efficient method must be developed to reduce effluent nutrients concentrations before this effluent is discharged. As part of a larger project evaluating biofilter effects on RAS effluent nutrient composition, this study focused on the effect of bivalve

culture on the composition of effluent from a southern flounder RAS. This system produces 1270L/day of enriched effluent, with nutrient and suspended solids concentrations 20–100× that of ambient levels. This effluent was introduced with seawater to an upweller system stocked with bivalves at two densities for 4–6 weeks. Oysters (*Crassostrea virginica*, average shell height =  $63.38 \pm 0.86$ ) and clams (*Mercenaria mercenaria*, average shell height =  $16.65 \pm 0.48$ ) were used in separate experiments. Effluent nutrient composition (total nitrogen, total phosphorus, and total suspended solids) was analyzed from samples (collected twice weekly) taken upstream and downstream of each upweller. Bivalve growth was quantified by measurement of shell height at the start and end of each trial. Analysis of preliminary data suggests that both oysters and clams exhibited growth over the course of the experiments. Water samples are currently being processed to determine the impact of bivalve activity on effluent nutrient composition.

**STATUS AND TRENDS OF BLACK ABALONE (*HALIOTIS CRACHERODII*): A PRELIMINARY APPROACH TO MODELING POPULATION VIABILITY.** Melissa Neuman<sup>1</sup>, John Butler<sup>1</sup>, Brian Tissot<sup>2</sup> and Daniel Goodman<sup>3</sup>. <sup>1</sup>National Marine Fisheries Service, <sup>2</sup>Washington State University Vancouver, <sup>3</sup>Montana State University.

Black abalone, *Haliotis cracherodii*, was added to the National Marine Fisheries Service (NMFS) species of concern list in 1999 because of documented large declines, known threats and uncertainty regarding the future course of those threats. Although efforts have been made to protect surviving populations in California, USA through closure of the commercial fishery in 1993, the species continues to decline and a >90% reduction in abundance has been observed throughout >50% of the species range since the late 1980s. Heavy fishing pressure and disease have contributed to the observed decline. Expanding coastal development may increase human traffic in the intertidal and may intensify enforcement problems. Pollution, warming temperatures, and biological interactions with competitors/predators may also threaten remaining populations. NMFS must determine whether the rate and extent of black abalone population decline and the severity of risk are high enough to warrant a listing under the United States Endangered Species Act. In order to do this, a variety of tools are employed using the best available data to predict the probability distribution of long-term population trends. A population viability model is one tool that is being developed to assess species vulnerability through the analysis of the interplay of specific demographic parameters over a spatial array of habitat suitability and other possible forcing factors. Our scientific challenge is to identify important gaps in our understanding of this species' ecology that will allow us to deter-

mine what data needs to be collected for improving the estimates of the most influential parameters.

**THE INFLUENCE OF EASTERN OYSTERS ON ECOLOGICAL PROCESSES IN CHESAPEAKE BAY: INSIGHTS FROM RECENT MODELING STUDIES.** R. I. E. Newell, Horn Point Laboratory, University of Maryland.

Results from mathematical models will be reviewed to examine how changes in eastern oyster abundance may affect benthic-pelagic coupling in Chesapeake Bay. All models indicate that at current abundances of ~1 adult oyster m<sup>-2</sup> on historical oyster bottom, oysters exert negligible influence on water quality. At abundances of ~10 oysters m<sup>-2</sup>, equivalent to the goal of the Chesapeake Bay Agreement of a 10-fold increase by 2010, models indicate that during warmer months oysters grazing on phytoplankton reduce turbidity to a level that significantly increases light availability for benthic plants. In cooler months, including during the spring phytoplankton bloom, oysters do not feed sufficiently actively to exert top-down grazer control. Biodeposition leads to appreciable removal of nitrogen through denitrification and burial. Resulting reduced phytoplankton biomass decreases microbial degradation of carbon beneath the pycnocline and increases oxygen concentration in bottom water. At ~100 oysters m<sup>-2</sup>, which is the estimated pre-colonial density, models indicate that oysters can exert a profound influence on water quality. Even though such ecosystem services are extremely valuable, the challenge is how to restore oysters in the face of relentless harvest pressure, disease epizootics, and the siltation of the cultch essential for larval settlement. Closure of the public fishery and the development of oyster aquaculture would help increase oyster abundances. An absence of leadership is preventing these necessary changes from occurring; indeed, management decisions often exacerbate the problem, such as the recent opening of Maryland oyster bars to the once illegal practice of oyster dredging.

**CHANGES IN SHELL STRENGTH OF *CRASSOSTREA VIRGINICA* AND *CRASSOSTREA ARIAKENSIS* IN RESPONSE TO CRAB PREDATORS FROM CHESAPEAKE BAY.** Roger I. E. Newell, Victor S. Kennedy and Kristi S. Shaw, Horn Point Laboratory, UMCES.

If *C. ariakensis* were to be introduced to Chesapeake Bay it is unknown if its abundance would be controlled by the same predators that affect *C. virginica* populations. In replicated laboratory studies, we compared the relative susceptibility of juvenile diploids (shell height <25 mm) of both oyster species to five species of potential crab predators. We tested two species of mesohaline

mud crab, *Rhithropanopeus harrisi* [carapace width 6–16 mm] and *Eurypanopeus demissus* [6–18 mm]), two species of polyhaline mud crab *Panopeus herbstii* [9–29 mm] and *Dyspanopeus sayi* [8–20 mm], and the euryhaline blue crab, *Callinectes sapidus* [35–65 mm]. We found that all four species of mud crab and the blue crab significantly ( $P < 0.05$ ) preferentially consumed *C. ariakensis* compared to *C. virginica*. We hypothesized that this differential predation may stem from differences in shell strength between the two species of oysters. Using an Instron load compression instrument we found that the strength of the upper valve of *C. ariakensis* was ~50% lower than for comparably sized *C. virginica*. Oysters of both species grown for 54 d in the presence of (but protected from) blue crabs exhibited different responses. *C. ariakensis* grew denser shells (total weight/shell area) that approximately doubled their compression strength compared to same species control oysters. The shell strength of *C. virginica* also approximately doubled, but these stronger shells had a significantly lower density than their controls. We conclude that both species of oysters respond morphologically to the presence of predators, although the response differs markedly between species.

**DEVELOPMENT OF LANDSCAPE MODELS FOR PROTECTION AND RESTORATION OF FRESHWATER MUSSELS IN LARGE RIVERS.** Teresa J. Newton<sup>1</sup>, Michelle R. Bartsch<sup>1</sup>, Jennie S. Sauer<sup>1</sup>, Jeff J. Steuer<sup>1</sup> and Steve J. Zigler<sup>2</sup>. <sup>1</sup>USGS, Upper Midwest Environmental Sciences Center, 2630 Fanta Reed Road, La Crosse, WI 54603, <sup>2</sup>USGS, WI District Office, 8505 Research Way, Madison, WI 53562.

We assessed whether the spatial distribution of mussels could be predicted from physical, hydraulic, and biologic variables in a large floodplain river. Analyses of data from mussel beds at small (0.4 km) and moderate (6 km) spatial scales indicated that computed hydraulic variables (e.g., shear stress, Froude number) were more predictive than simple, measured variables (e.g., depth, velocity, substrate particle size). Similarly, classification tree models of mussel presence-absence in a 38-km reach of the river, which had a prediction success of ~75%, were largely driven by shear stress and substrate stability, but interactions with simple physical variables (e.g., slope) were also important. Moreover, discharge-specific models suggested that episodic events such as droughts and floods were more important in structuring mussel distributions than conditions during average flows. Because mussel species differ in shell features that may influence displacement during high discharge events, we evaluated if sculptured, thick-shelled species occupied areas with higher hydraulic stresses, compared to non-sculptured, thin-shelled species. Classification tree models predicted the presence of all mussels, regardless of shell morphology, in areas with low to moderate hydraulic stresses under low and high flow conditions. Overall, our studies suggest that mussels are

influenced by a complex interaction of biotic and abiotic factors acting at various spatial scales, but that certain hydraulic variables can improve our ability to predict their spatial distribution in large rivers.

**UNIONID FEEDING STRATEGIES.** S. J. Nichols, U. S. Geological Survey, 1451 Green Rd., Ann Arbor, MI, 48105.

The continued decline of many unionid communities throughout North America has been attributed to many factors ranging from pollution to exotic species. However, one main factor, food supplies, is rarely discussed. This raises a question: are populations declining in some locations because critical food supplies are no longer present? A review of the current research on unionid feeding highlights our limited knowledge about dietary requirements. Feeding studies on wild populations have been limited because of the difficulties in separating assimilation from ingestion in filter-feeding organisms. Recent technological advances such as stable isotopes and  $C^{13}$  enrichment have provided some information on food selectivity and have shown that unionids feed on a wide range of items including algae, dissolved organic molecules and bacteria. But these studies have not detected any food resource partitioning between sympatric genera or species. All species, regardless of shell length, type of gill cirri, siphon structure, or physical location in the substrate, appear to feed on particles <29 microns and can obtain food from either planktonic or benthic sources. However, such apparent omnivory does not translate into easily-maintained adults under captive conditions. Without access to natural food supplies, adult unionids rarely thrive. Dietary supplements such as used for marine bivalves do not support adult survival, though larvae can be reared using marine algae. Apparent omnivory is obviously misleading regarding actual dietary needs. In-depth surveys of potential food resources in waters with healthy unionid populations are critically needed to understand the relationship between food resources, unionid feeding strategies, and long-term survival.

**PHYSIOLOGICAL BASES OF GENETICALLY DETERMINED VARIATION IN GROWTH OF BIVALVE LARVAE (*CRASSOSTREA GIGAS*).** Douglas A. Pace<sup>1</sup>, Adam G. Marsh<sup>2</sup>, Patrick Leong<sup>3</sup>, Allison Green<sup>3</sup>, Dennis Hedgecock<sup>3</sup> and Donal T. Manahan<sup>3</sup>. <sup>1</sup>AHF-210, Department of Biological Science, University of Southern California, Los Angeles, CA, 90089, <sup>2</sup>University of Delaware, <sup>3</sup>University of Southern California.

Variation in growth rates has been well documented in larval stages of bivalve molluscs. The endogenous processes that might contribute to individual-based variations in growth rates are not well understood. In this study, we used inbred lines of the Pacific

oyster, *Crassostrea gigas*, in a series of factorial crosses to produce families of larvae with different growth rates that were genetically set, even under identical environmental conditions of food and temperature. Growth rates of these larval families varied over a 5-fold range, from 3.4 to 17.6  $\mu\text{m day}^{-1}$ . A suite of integrated measurements were applied to study growth variation in larvae, including: size, biochemical compositions, rates of acquisition of both dissolved and particulate nutrients, absorption efficiencies, metabolic rates, and enzyme activities. Fast-growing larvae had similar size-specific metabolic rates and capacities to slow-growing larvae, but the fast-growing larvae had significantly higher size-specific feeding rates. Also evident was the complex regulation of enzymatic activities responsible for ion transport (a metabolically expensive process in animals). Our results show that while feeding rates were important in explaining a portion of the growth differences, the regulation and internal allocation of metabolic energy played a large role in genetically-set growth rate variation. The larvae studied here clearly exhibited the phenomenon of hybrid vigor ("heterosis"). Our results will be discussed in the context of understanding the physiological bases of growth heterosis.

**INVASION OF *CRASSOSTREA GIGAS* IN MARINE RESERVES: LOCAL REPRODUCTION OR DISPERSAL FROM ELSEWHERE?** Dianna K. Padilla<sup>1</sup> and Terrie Klinger<sup>2</sup>. <sup>1</sup>Stony Brook University, <sup>2</sup>University of Washington.

The Pacific oyster, *Crassostrea gigas*, has been an important aquaculture species in the Pacific Northwest for >75 years. Because this species requires waters 19–20 °C for spawning, it has been considered a safe introduction, without the possibility of escape due to local reproduction limits. However, *C. gigas* has been invading rocky shores in the San Juan Archipelago, Washington for almost a decade, and is more abundant within than outside marine reserves. This recent invasion could be due to: 1) changes in temperature such that spawning can be induced, 2) thermal loading during low tide (mid-day) that allows oysters to spawn even though the water is cold, 3) evolution for spawning at lower temperatures, or 4) spawning or larval release elsewhere and dispersal into marine reserves and adjacent shores. We tested among these hypotheses by monitoring local temperatures and experiments to determine if oysters collected from the field will spawn when held at ambient water temperatures or at ambient water temperatures when exposed to a tidal cycle. We also tested whether larvae produced by local *C. gigas* were capable of development in the cold waters typical of the San Juans. These oysters do not appear able to spawn at temperatures <17–18 °C, and larval development even at 15 °C is slowed >3 times from development at 20 °C. Even in quiet bays, temperatures are not warm enough to

induce spawning. These data suggest that larval production elsewhere must be responsible for this invasion.

**EVALUATION OF THE ANTIMALARIAL DRUG QUININE AS A POTENTIAL CHEMOTHERAPEUTIC AGENT FOR THE EASTERN OYSTER PARASITE, *P. MARINUS*.** Christina Panko, Aswani Volety, Vincent Encomio and Jose Barreto. Florida Gulf Coast University, 10501 FGCU Blvd South, Ft. Myers, FL 33965.

Currently no anti-protozoal drug therapy exists in aquaculture to effectively treat or prevent *Perkinsus marinus* infections of the eastern oyster. Development of a chemotherapeutant specific for treating oysters infected with *P. marinus* or preventing disease may be an additional measure to avert physiological strain on aquaculture brood stock before spawning. Given the taxonomic proximity of *P. marinus* to Apicomplexans such as the malarial parasite, *Plasmodium* spp, the antimalarial drug Quinine was screened for drug sensitivity of cultured *P. marinus*. Following an acute challenge with varying concentrations of Quinine HCl, the viability of one strain of cultured meronts isolated from Laguna Madre, Texas oysters was determined using a dye reduction method. After a three hour exposure, the highest concentration tested, 50  $\mu\text{g/ml}$ , had the most significant effect ( $p = 0.000$ ) on parasite viability. Following assay optimization, six geographically distinct *P. marinus* isolates (ranging from Connecticut, New Jersey, Maryland, Virginia, Louisiana, and Texas) were exposed to 50  $\mu\text{g/ml}$  Quinine HCl to evaluate differences in susceptibility. The viability of all isolates significantly decreased after Quinine exposure. The Maryland and Virginia strains appeared to be more susceptible to treatment than the other isolates. Bearing in mind that hemocytes are integral component of the host innate immune system, the effect of Quinine on hemocyte viability was determined using a Neutral Red assay. The resulting information indicated the importance of examining the use of Quinine in mitigating *P. marinus* infections under *in vivo* conditions.

**ESTABLISHING BASELINES FOR RECOVERY OF OYSTER (*CRASSOSTREA VIRGINICA*) POPULATIONS IN SOUTHEAST FLORIDA.** Melanie L. Parker and William S. Arnold. Fish & Wildlife Research Institute, 100 Eighth Ave. SE, St. Petersburg, FL 33701.

Oysters occur in nearly all estuarine and nearshore waters along the Florida coast. Many of these waters have experienced altered patterns of water delivery and quality as a result of water management practices related to the St. John's River and Kissimmee River basins, Lake Okeechobee and the Everglades. Alterations in

freshwater flow have reduced or eliminated many oyster reefs and impacted both the timing and extent of oyster reproduction. The Comprehensive Everglades Restoration Program (CERP) is being implemented as a means of reinitiating natural freshwater flow to coastal waters in south Florida. Because of its wide distribution and essential habitat value, the eastern oyster is included in this program as a target species for monitoring and restoration. Changes in oyster distribution and abundance, reproductive development, disease incidence, and juvenile recruitment and growth are being monitored at several sites along the southeast coast of Florida including the St. Lucie estuary, Loxahatchee River, Lake Worth Lagoon and Biscayne Bay. Oyster populations in Tampa Bay, Mosquito Lagoon and Sebastian River are also being monitored as a background for comparison because these are outlier populations not affected by CERP water management practices. In January 2005, study sites were established within each estuary and monthly sampling of recruitment, reproductive development and disease incidence was initiated. Adult surveys were also conducted at each study site to determine oyster distribution and abundance. Preliminary results indicate that the pattern of degradation of oyster populations among estuaries is rather chaotic while the pattern within an estuary is more predictable.

**IMPACTS OF THE TOXIC DINOFLAGELLATE *ALEXANDRIUM MONILATUM* ON THREE ECOLOGICALLY IMPORTANT SHELLFISH SPECIES.** Susan E. Pate<sup>1</sup>, JoAnn M. Burkholder<sup>1</sup> and Sandra E. Shumway<sup>2</sup>. <sup>1</sup>North Carolina State University, <sup>2</sup>University of Connecticut, Groton, CT.

Little is known regarding interactions between shellfish and *Alexandrium monilatum*, a toxigenic dinoflagellate species that forms blooms mostly in the Gulf of Mexico. Toxic *A. monilatum* has been linked to fish and invertebrate kills, and produces endotoxins with hemolytic and neurotoxic properties. We experimentally assessed responses of three ecologically important shellfish species to *A. monilatum* (toxic strain AMO3, NOAA National Ocean Service, Charleston, SC). In the first set of experiments, grazing studies were conducted with adult and juvenile eastern oysters (*Crassostrea virginica*), northern quahogs (*Merccenaria mercenaria*), and green mussels (*Perna viridis*), which inhabit areas where *A. monilatum* blooms occur. Clearance rates of each shellfish species were depressed when exposed to toxic *A. monilatum* (bloom density of ~550 cells/mL) alone or with nontoxic Instant Algae® *Paylova*, in comparison to clearance rates of control animals that were fed a nontoxic dinoflagellate of similar size, *Alexandrium tamarense* (nontoxic clone CCMP115) and benign cryptophyte algae. Exposure to *A. monilatum* also significantly decreased shellfish valve gape. In the second set of experiments, bioassays were conducted to test survival of larval shellfish (*M. mercenaria*, *C. virginica*) exposed to *A. monilatum* as whole, unconstrained cells, cells held in dialysis tubing, or sonicated cells. Sonicated *A. monilatum* caused a significant decrease in larval

survival, in comparison to survival of the control larvae that were tested with nontoxic *A. tamarense*. Overall, these data indicate that *A. monilatum* blooms can adversely affect shellfish survival by reducing clearance rate and valve gape, affecting food intake, and inducing larval mortality.

**OBSERVATIONS ON THE REPRODUCTIVE BIOLOGY OF THE HAIRY CRAB *HAPALOGASTER MERTENSII*, ON KODIAK ISLAND, ALASKA.** Susan A. Payne, Jan A. Haaga and Bradley Stevens. AFSC NOAA Fisheries, Kodiak Fisheries Research Center, 301 Research Ct., Kodiak, AK 99615.

The reproductive biology of female *Hapalogaster mertensii*, an intertidal lithodid crab, from Kodiak Island, Alaska was observed in both the laboratory and field from 2000–2004. Larval release, in 2001, 2003, and 2004, occurred from February–June in the laboratory, and was not significantly different between years. Hatch duration in the individual crabs varied significantly between years and pairwise comparisons showed the following significant differences between years: 2001 ( $32.38 \pm 3.37$  days) > 2003 ( $22.62 \pm 2.08$  days) > 2004 ( $6.50 \pm 3.37$  days). The average number of larvae released was  $214.75 \pm 37.66$  in 2001 and  $157.38 \pm 23.24$  in 2003, and was not significantly different. Molting, mating, and the extrusion of a new clutch of eggs are not contiguous as in other lithodid crabs, but occur over a period of two to five months. Laboratory observations show that molting occurs twice in some females between hatching and extrusion. Possible mating behavior was observed twice, and occurred after the first molt, but was not associated with the molt or extrusion of new eggs. Egg extrusion in the lab was only observed in 2003 and occurred between 10 August and 10 November, and in the field in 2002 as early as July. Ovary incubation between the crabs was significantly different ranging from 187 to 244 days ( $224.67 \pm 8.4288$ ,  $P = 0.00$ ). Lab conditions appeared to mimic field observations for the timing of larval release and egg extrusion despite prolonged captivity of lab animals.

**TRIPOID *CRASSOSTREA ARIAKENSIS* AND *CRASSOSTREA VIRGINICA* GROWN AT FOUR SITES IN CHESAPEAKE BAY.** Kennedy Paynter<sup>1</sup>, Donald Meritt<sup>2</sup>, Stan Allen<sup>3</sup>, Jake Goodwin<sup>1</sup> and Marey Chen<sup>1</sup>. <sup>1</sup>University of Maryland, <sup>2</sup>University of Maryland Center for Environmental Science, <sup>3</sup>Virginia Institute of Marine Science.

Triploid suminoe oysters, *Crassostrea ariakensis*, and triploid eastern oysters, *Crassostrea virginica*, were produced nearly simultaneously in the hatchery and deployed in cages at four sites in Chesapeake Bay in April 2004. The four sites represented the broad range of salinities present in the Bay. The 'high' salinity site was in the York River near the Virginia Institute of Marine Science (VIMS; mean salinity 19 psu). The Patuxent River site had a mean salinity of 11 psu and the Choptank River site of 9 psu. The 'low' salinity site in the Severn River had a mean salinity of 6 psu.



Growth of both species was positively correlated with salinity. As of September 2004, no significant differences in size or disease prevalence existed between species at any site. Dermo, the disease caused by *Perkinsus marinus*, was detected in both species in July but only at the York River site. However, *C. ariakensis* grew more later in the year than did *C. virginica* and appeared to begin growing earlier in the following spring. Thus, *C. ariakensis* became significantly larger by June 2005. During the summer of 2005, *C. virginica* at the Choptank and York River sites grew more slowly than *C. ariakensis*, presumably due to *P. marinus* infections. Mean shell height of *C. ariakensis* at the York River site in October 2005 (deployed 19 months) was 140 mm compared to 105 mm in *C. virginica*. Physiological differences between the species apparently allow *C. ariakensis* to grow more actively at lower temperatures.

**USING PCR TO DETERMINE THE ACCURACY OF MORPHOLOGICAL IDENTIFICATION OF *MERCENARIA MERCENARIA* LARVAE.** Laurie Perino, Dianna Padilla and Michael Doall, Stony Brook University, Stony Brook, NY.

Identification of bivalve larvae in the field is an important aspect of fisheries management, restoration efforts and early warnings of invasive species. Traditional methods of identifying bivalve larvae are based on morphological characteristics of the larval shell. Problems occur when using this method because the larvae of many different species are very similar in shape and size, especially during the smaller D-stage. Newer molecular methods, including multiplex PCR, have been developed to aid in larval identification. The study presented here discusses the accuracy of morphological identification and provides error rates for misidentified larvae by using PCR to identify individual field collected larvae to species. For larvae suspected to be *Mercenaria mercenaria*, the rate of obtaining a false negative (identifying a larva as another species when it is actually *M. mercenaria*) was very low; however the rate of obtaining a false positive (identifying a larva as *M. mercenaria* when it is actually another species) was quite high. These error rates suggest that morphological characteristics are helpful in determining those larvae that are not the target species, but may lead to overestimations of densities of the target species due to overlaps in size and shape of different species.

**ENRICHED ARTEMIA NAUPLII AS DIET FOR RED (*PARALITHODES CAMTSCHATICUS*) AND BLUE (*P. PLATYPUS*) KING CRAB LARVAE IN THE LABORATORY.** Sara Persselin, NOAA Fisheries, Kodiak Fisheries Research Center, 301 Research Ct., Kodiak, AK 99615.

Newly-hatched unenriched *Artemia* nauplii are often the primary diet in culturing red (*Paralithodes camtschaticus*) and blue (*P. platypus*) king crab larvae in the laboratory. Although this diet allows survival to the first juvenile crab stage (C1), rates are sub-optimal. This work reports on studies over the last two years

investigating whether diets of enriched *Artemia* nauplii improve survival of red (RKC) and blue (BKC) king crab larvae. In 2003, RKC larvae were fed one of seven diets: (1) newly-hatched unenriched *Artemia*, (2–5) *Artemia* nauplii enriched with the microalga *Rhodomonas salina*, *Isochrysis* sp., *Thalassiosira nordenskiöldii*, or *T. aestivalis*, or (6–7) *Artemia* nauplii enriched with a commercial enrichment product, *Isochrysis* paste or Algamac 3050. The treatment that resulted in the highest survival to the non-feeding glaucothoe stage was *T. nordenskiöldii* (73%) and the treatment that resulted in the highest survival to C1 was *Isochrysis* paste (63%). Lowest survival to both stages occurred in the newly-hatched nauplii treatments (42% and 30%). Based on these results, BKC larvae were fed one of four diets in 2004: 1) *Artemia* nauplii enriched with the diatom *T. nordenskiöldii*, 2) *Artemia* nauplii enriched with frozen *Isochrysis* paste, 3) newly-hatched unenriched *Artemia* with *T. nordenskiöldii* added to the culture water and 4) no food. The treatment that resulted in the highest survival through C1 was newly-hatched *Artemia* with *T. nordenskiöldii* added to the culture water (91.7%). This diet is superior to either enriched *Artemia* nauplii or unenriched newly-hatched *Artemia* for king crab larvae cultured in the laboratory.

**SITE-SPECIFIC GROWTH RATES OF ATLANTIC SURF CLAMS, *SPISULA SOLIDISSIMA*, FROM THE MIDDLE ATLANTIC BIGHT.** Adriana Picariello, Roger Mann and Juliana M. Harding, The Virginia Institute of Marine Science, P.O. Box 1346, Gloucester Point, VA 23062.

In recent years, the general distribution of the Atlantic Surfclam, *Spisula solidissima*, along the east coast of the USA has undergone a dramatic shift both northward and into deeper water. This observation is based on 20 years of NOAA/NMFS/NEFSC stock assessments, with sampling from 1999 to the present indicating that this distribution shift has occurred. We examined surfclam growth from different locations along the current distribution. These locations included animals from both the southern and northern areas within the distribution as well as both inshore and offshore locations within these areas. Samples included a size range of animals 32 mm to 180 mm in shell length. Surfclam ages were determined by counting growth bands in the chondrophore. We present and compare estimates of age structure and growth from surf clam populations along a latitudinal and bathymetric gradient. Fitted growth curves were compared across and within sites.

**TEMPORAL EXPRESSION OF GENETIC LOAD IN TWO FAMILIES OF THE PACIFIC OYSTER *CRASSOSTREA GLIGAS*.** Louis Plough, University of Southern California.

Discovery of a large number of deleterious recessive mutations (genetic load) in the Pacific oyster (Launey & Hedgecock 2001, Genetics 159:255) explains distorted segregation ratios, seen widely in bivalves, and supports the dominance theory of heterosis



and inbreeding depression, for which there is substantial evidence. However, the fine scale mapping location and temporal expression of these deleterious alleles has not been well characterized. Previous experiments to map genetic load in the Pacific oyster had relatively poor genomic coverage and analyzed only two time points. We are attempting more complete genome coverage, based on a consensus microsatellite linkage map, evaluating over 90 markers and sampling larvae every day for thirty days post fertilization. Using inbred lines from wild populations and classical crossbreeding experiments, we are analyzing segregation ratios for 50 informative microsatellite DNA markers in larvae at 30 days post fertilization from an  $F_2$  and  $F_3$  hybrid family. We look for deviations from expected Mendelian inheritance ratios, owing presumably to selection against identical-by-descent homozygotes linked to nearby markers, and apply a linked selection model to estimate the number of lethal genes in the wild founders. We see, for example, one distortion on linkage group 9, where microsatellite *ucdCgi189* has only three homozygous genotypes of an expected 18 ( $N = 66$ ). We are then following the distorted markers back in time to determine when the deleterious alleles to which they are linked were first expressed in development.

**MEASURING SUCCESS IN OYSTER REEF RESTORATION: APPLICATION OF STANDARDIZED MEASURES AND TESTS OF APPROACHES.** Martin H. Posey<sup>1</sup>, Troy D. Alphin<sup>2</sup>, Loren Coen<sup>3</sup>, Keith Walters<sup>4</sup> and Dara Wilber<sup>5</sup>. <sup>1</sup>Department of Biology and Marine Biology, UNC-Wilmington, <sup>2</sup>Center for Marine Science, UNC-Wilmington, <sup>3</sup>Marine Resources Research Institute, SCDNR, <sup>4</sup>Department of Marine Science, Coastal Carolina University, <sup>5</sup>Grice Marine Program, College of Charleston.

Oysters along the Atlantic and Gulf coasts of North America have supported a historically important commercially fishery. Oysters are also increasingly recognized as key components of coastal ecosystems, providing habitat for transient and resident fauna, affecting particulate concentrations in overlying waters, reducing wave energy along sensitive shorelines and influencing local biogeochemical cycling. With a decline in oysters along much of the Atlantic coast, there is increased attention to restoring reefs through shell planting efforts. However, the lack of long-term monitoring using standardized methodologies that target both population and ecosystem functions has been an impediment to assessing the success of many restoration projects. In 2004, researchers at a SeaGrant-sponsored workshop proposed a set of sampling criteria and methodologies to provide standardized population and ecosystem measures for assessing the success of oyster restoration projects. We are applying these standardized measures to a series of restored reefs of varying ages along the central and southeast North Carolina coast. This includes comparison of selected habitat and population functions to reference reefs as well as examining the influence of seeding (placing hatchery oysters on

the reef at the time of creation), varying tidal position (intertidal to shallow subtidal), and landscape attributes, such as shell depth and proximity of adjacent habitat types, in reef establishment. This project will provide a comparison among different restoration designs as well as providing information on habitat function in a standardized form that can be compared among studies.

**UNDERSTANDING *CRASSOSTREA GIGAS* POPULATION VARIABILITY USING A GENETICS-BASED MODEL.** Eric N. Powell<sup>1</sup>, John M. Klinck<sup>2</sup>, Dennis Hedgecock<sup>3</sup> and Eileen E. Hofmann<sup>2</sup>. <sup>1</sup>Haskin Shellfish Research Laboratory, Rutgers, The State University of New Jersey, 6959 Miller Ave., Port Norris, NJ 08349, <sup>2</sup>Old Dominion University, <sup>3</sup>University of Southern California.

Survival and recruitment of marine bivalve larvae represent the interplay of environmental, biological, and physiological processes. However, the genetic characteristics of individual members of a population provide an overlay that determines the overall population response to exogenous and endogenous forces. This study describes the first genetically-based model developed for a marine bivalve species, *Crassostrea gigas*, that can be coupled to more traditional models describing animal growth and development. Explicit inclusion of genetic structure allows a mechanistic understanding of variability in growth, survival, and recruitment. The individual-based genetics model simulates generational changes in population genotype as a function of the number of parents and their genotypes. Each individual has 10 pairs of chromosomes, with multiple genes per chromosome and up to four alleles per gene. Some genes affect fitness by varying the chance of mortality; among these are homozygous recessives that are always lethal. The model structure allows genetic mutation and recombination, tracks male and female individuals by means of a sex gene, and permits the effective number of broodstock to be a small percentage of the total adult population. Multi-generational simulations show the disappearance of alleles, a decrease in heterozygosity, and a low effective population number, when only a few parents produce successful progeny and maintain the population yearly. Predicted sex ratios match observations, including the shift from male to female dominance with increasing size. Homozygous recessive genes develop in the population through mutation and slowly accumulate until common enough to affect population dynamics, which then restrains further increases.

**COPPER IS AN ESSENTIAL NUTRIENT AND POTENTIAL TOXICANT IN THE DIET OF THE SEA URCHIN (*LYTECHINUS VARIEGATUS*).** Mickie L. Powell<sup>1</sup>, W. T. Jones<sup>1</sup>, V. K. Gibbs<sup>1</sup>, H. S. Hammer<sup>1</sup>, J. M. Lawrence<sup>2</sup> and A. L. Lawrence<sup>3</sup>. <sup>1</sup>University of Alabama at Birmingham, Birmingham, AL, <sup>2</sup>University of South Florida, <sup>3</sup>Texas A&M University System.

Due to worldwide overfishing, scientists are currently developing stock enhancement and aquaculture techniques for sea urchins. Successful culture of sea urchins requires a formulated feed

that fulfills all nutritional requirements. Copper is an essential micronutrient in the diets of most animals. It is a component of many enzymes involved in energy production and in the protection of cells from free radicals. Excessive levels in the diet can be toxic. Small (ca. 13 g wet weight) *L. variegatus* were fed formulated feeds with low, medium, or high levels of copper for 12 weeks (calculated at 6, 47 and 160 ppm; levels based on established dietary levels for other marine invertebrates, supplemented as  $\text{CuSO}_4$ ). Under these experimental conditions, wet weights of individuals fed a medium level of copper were slightly higher ( $43.2 \pm 1.2$ ;  $P = 0.069$ ) than those fed low and high levels ( $39.9 \pm 1.2$ , and  $40.3 \pm 1.7$  g wet weight, respectively). Gonad and gut wet weights were significantly lower ( $P < 0.003$ ) in the high copper diet ( $6.9 \pm 2.5$ ,  $7.0 \pm 3.6$  and  $5.0 \pm 1.6$  g gonad wet weight and  $0.97 \pm 0.04$ ,  $1.1 \pm 0.06$  and  $0.83 \pm 0.04$  g gut wet weight for low, medium and high levels, respectively). However, in the absence of copper supplementation survivorship decreased to 87.5% on the low copper feed. These data suggest that dietary copper is essential for normal physiological function, but can be toxic at high levels.

**VIBRIO PARAHAEMOLYTICUS IN ALASKA: AN AGGRESSIVE RESPONSE TO A POTENTIAL CRISIS.** Raymond RaLonde, Alaska Sea Grant Program, University of Alaska, Anchorage, AK 99501.

Oyster farmers in Prince William Sound, during the summer of 2004, were devastated by an outbreak of human illness caused *Vibrio parahaemolyticus* (*Vp*). With 63 confirmed cases, the outbreak was the second largest recorded in the United States history.

The United States Food and Drug Administration (FDA) is concerned about the Prince William Sound outbreak. The first serious problem is that the O6:K18 serotype, while similar to an isolate from the state of Washington, is extremely virulent, causing illness in concentrations as low as 3.5 bacteria per gram. In addition, over 76% of the environmental samples, tested positive for pathogenic *Vp*, while the worldwide historical averages less than 1%. Both of these factors indicate an unprecedented event that may have nationwide implications in further development of seafood safety standards.

As a follow-up to the 2004 outbreak, a statewide investigation involving the industry, Alaska Department of Environmental Conservation, FDA Gulf Coast Shellfish Laboratory, and the University of Alaska Sea Grant Program was implemented during the summer of 2005. The objectives of the study were to determine the extent of *Vp* in environmental samples in Prince William Sound, determine if *Vp* was present at oyster farms outside of Prince William Sound, and to test changes in aquaculture practices as preventative measures against *Vp* accumulation. Preliminary results indicate that *Vp* is broadly distributed in environmental

samples in Prince William Sound, and prompt intervention and temperature control was effective in preventing *Vp* illness from culture Pacific oysters.

**MICROSATELLITE ASSESSMENT OF GENE FLOW IN AMBLEMA PLICATA IN THE OUACHITA HIGHLANDS OF SOUTHEASTERN OKLAHOMA.** Kathleen Reagan<sup>1</sup> and Caryn Vaughn<sup>2</sup>. <sup>1</sup>University of Oklahoma, Oklahoma Biological Survey, <sup>2</sup>University of Oklahoma, Director-Oklahoma Biological Survey.

Appreciation of genetic diversity in unionids is essential to their preservation. Understanding how habitat perturbations, particularly those that fragment populations, affect this genetic diversity will provide information for better management of their natural habitats. We are examining genetic diversity and relatedness within a common mussel species, *Ambelma plicata*, from three rivers in the Ouachita Highlands of southeastern Oklahoma that are fragmented to varying degrees, due to impoundments. We sampled *A. plicata* tissue from ten sites across the Little ( $N = 8$ ), Glover ( $N = 1$ ) and MT. Fork ( $N = 1$ ) Rivers. At each of these sites, mussels were sampled from 15, randomly placed, 0.25 m<sup>2</sup> quadrats. Tissue samples consisting of 20–40 µg of mantle tissue were clipped from a minimum of 30 individuals per site. A subsample of mussel individuals was taken from each site for shell thin-sectioning to determine age. Microsatellite markers will be used to examine both genetic diversity and relatedness of *A. plicata* within and between sites. This will enable us to determine the impacts of impoundments on gene flow.

**MOLECULAR MARKERS FOR POPULATION STUDIES, SELECTIVE BREEDING AND PARENTAGE ASSIGNMENTS IN THE HARD CLAM (MERCENARIA MERCENARIA).** Kimberly S. Reece<sup>1</sup>, Gail P. Scott<sup>1</sup>, Ryan B. Carnegie<sup>1</sup>, Lisa Ragone Calvo<sup>2</sup>, Eugene M. Bureson<sup>1</sup> and Mark D. Camara<sup>3</sup>. <sup>1</sup>VIMS, The College of William and Mary, P.O. Box 1346, Gloucester Point, VA 23062, <sup>2</sup>Haskin Shellfish Research Laboratory, Rutgers University, <sup>3</sup>USDA, ARS, OSU, Hatfield Marine Science Center.

The hard clam (*Mercuraria mercenaria*) industry has been heavily impacted by Quahog Parasite Unknown (QPX), which had caused substantial mortality in cultured clams at some sites along the eastern seaboard of the USA. Selective breeding of clams for traits such as disease resistance, enhanced growth rate and low salinity tolerance might provide stocks that significantly improve harvest yields in particular regions. Stocks derived from geo-

graphically distinct wild broodstock demonstrate differential susceptibility to QPX, suggesting a genetic basis for disease resistance. Side-by-side field experiments have shown significantly higher QPX prevalence in stocks from Florida and South Carolina compared to that observed in northern stocks (VA, NJ, MA). Unfortunately, genetic improvement efforts in bivalves are hampered by difficulties inherent in maintaining individually reared families in order to address genetic questions, and in developing elite genetic stocks without severe inbreeding depression. Molecular genetic markers can be used to assign individuals to families following field grow-out challenges and to facilitate maintenance of genetic diversity in stocks. For disease selection challenges, families can be mixed in the field and multi-locus genotypes can be used to assign individual progeny to parents or to reconstruct sibling relationships retrospectively. Individuals used for subsequent generation spawns can be selected based on genetic profiles to maximize diversity and genetic profiles of populations and stocks allow examination of genetic differentiation. We have developed both microsatellite and single nucleotide polymorphism (SNP) markers for *M. mercenaria* and are testing them for the ability to do parentage assignments and stock differentiation.

**GENETIC CONSIDERATIONS IN BROODSTOCK SELECTION FOR OYSTER REEF RESTORATION.** Kimberly S. Reece<sup>1</sup>, Jens Carlsson<sup>1</sup>, Jan F. Cordes<sup>1</sup>, Ryan B. Carnegie<sup>1</sup> and Mark D. Camara<sup>2</sup>. <sup>1</sup>VIMS, The College of William and Mary, P.O. Box 1346, Gloucester Point, VA 23062, <sup>2</sup>USDA, ARS, OSU, Hatfield Marine Science Center.

Diseases caused by the parasites, *Perkinsus marinus* and *Haplosporidium nelsoni*, contributed to decline of native *Crassostrea virginica* populations in the eastern USA, and slowed their recovery. Recently, oyster restoration strategies have focused on improving tolerance to these parasites by planting selectively bred strains in Chesapeake Bay. Enhanced parasite tolerance in domesticated stocks might translate to increased chances of survival and reproductive success relative to wild populations. In addition, disease resistance genes from selected strains might introgress into wild populations through interbreeding. Genetic variation, however, is typically greatly reduced in hatchery lines compared to natural populations and depending on genotypic profiles, as well as relative census and effective population sizes, interbreeding with planted hatchery strains could reduce diversity in wild populations. Oyster populations in the Bay might become genetically less resilient in the face of future disease or environmental challenges. Understanding the genetic mechanisms of disease resistance and interactions between deployed hatchery stocks and wild populations is key to assessing the impact, positive or negative, of such oyster restoration strategies. Many hatchery stocks being used for restoration in Chesapeake Bay have unique genetic profiles dis-

tinguishing them from local wild populations. We are using molecular markers to evaluate the breeding success of deployed oysters and conducting surveys to examine the impact of disease on various populations and selected stocks. Genotypic data enables us to assign individuals to either local native populations, deployed oyster strains or putative hybrid groups, and disease data provides information on relative disease tolerances of the different groups.

**REPRODUCTIVE CYCLE OF THE RAZOR CLAM *SOLEN MARGINATUS* (PULTENEY 1799) IN SPAIN. A COMPARATIVE STUDY IN THREE DIFFERENT PLACES.** A. Remacha-Triviño, University of Rhode Island, Department of Fisheries, Animal and Veterinary Sciences, 20A Woodward Hall, RI 02881.

The reproductive cycle of the razor clam, *Solen marginatus*, (Pulteney 1799) is studied in three different places of Spain through time. Sampling was performed in the natural beds of: Eo Estuary (NW Spain) in 1994–95, Santander Bay (N Spain) in 1998–99 and Terrón Estuary (SW Spain) in 1999. In Eo, Santander and Terrón, respectively: phase 0 (resting stage) occurred in July–August, September–October and August–September, phase I (early gametogenesis) happened in: August–October, October–December and September–November, phase II (late gametogenesis) was developed in November–April, January–May and December–April and phase III (reproduction) took place in: May–June, June–August and May–July. Two new approaches oriented to the fast monitoring of the gametogenic cycle of the species based on sequential mean drained soft parts weights obtained by simulation and on the macroscopic observation of the gonad are presented.

**QUANTIFICATION OF *PERKINSUS MARINUS* IN THE EASTERN OYSTER *CRASSOSTREA VIRGINICA* USING MODERN STEREOLOGICAL TECHNIQUES.** Antonio Remacha-Triviño<sup>1</sup>, Dodi Borsay-Horowitz<sup>2</sup>, Christopher Dungan<sup>3</sup>, Ximo Gual-Arnan<sup>4</sup>, Javier Gómez-León<sup>1</sup> and Marta Gómez-Chiarri<sup>1</sup>. <sup>1</sup>University of Rhode Island, 20A Woodward Hall, Kingston, USA, <sup>2</sup>US EPA, Atlantic Ecology Division, <sup>3</sup>Maryland DNR, Cooperative Oxford Laboratory, <sup>4</sup>Universitat Jaume I, Castelló, Spain.

Dermo disease, caused by the protozoan parasites *Perkinsus* spp., is responsible for high mortalities of bivalve molluscs worldwide. In order to improve the knowledge of the pathogenesis of Dermo, accurate techniques to estimate the number of parasites in tissue sections are required. This study is aimed to quantify the number and tissue distribution of different stages of *P. marinus* in a natural population of *Crassostrea virginica* from Wickford

(Rhode Island, US) by the application of modern stereology and immunohistochemistry. Mean total number of trophozoites in eight oysters collected in July 2005, of mean shell length (mean  $\pm$  SD) of  $101.2 \pm 5.2$  mm, were (mean  $\pm$  SE)  $11.80 \pm 3.91$  million and  $11.55 \pm 3.88$  million for the optical disector and optical fractionator methods respectively. The mean empirical error between both stereological approaches was  $3.8 \pm 1.0\%$ . Trophozoites were detected intracellularly in the following tissues: intestine (30.1%), Leydig tissue (21.3%), hemocytes (14.9%), digestive gland (11.4%), gills (6.1%), connective tissues (except Leydig and mantle) (5.7%), gonads (4.1%), palps (2.2%), muscle (1.9%), mantle connective (0.8%), pericardium (0.7%), mantle epithelium (0.1%), and heart (0.1%). The remaining 0.6% of trophozoites were found extracellularly throughout different tissues. Percentages of trophozoite stages were (mean  $\pm$  SE): large, log-phase trophonts (*i. e.* signet-rings):  $97.0 \pm 1.2\%$ ; meronts:  $2.0 \pm 0.9\%$ ; clusters of small, log-phase trophonts (*i. e.* merozoites):  $1.0 \pm 0.5\%$ . These techniques could be useful to follow parasite distribution and progression in experimental infections, and further explore mechanisms of dermo pathogenesis.

#### HERPES VIRUSES INFECTING BIVALVES: A REVIEW. T. Renault, IFREMER, La Tremblade, France.

Particles morphologically similar to herpes viruses were first detected in an invertebrate (the Eastern oyster, *Crassostrea virginica*) in 1972. Herpes-like viruses have since been identified in various marine bivalve species throughout the world, including the Pacific oyster, *Crassostrea gigas*, the European flat oyster, *Ostrea edulis*, the Antipodean flat oyster, *Ostrea angasi*, the Chilean oyster, *Tiostrea chilensis*, the carpet shell clam, *Ruditapes decussatus*, the Manila clam, *Ruditapes philippinarum* and the great scallop, *Pecten maximus*. Infections are often associated with sporadic episodes of high mortality among larvae and juveniles. PCR-based diagnostic methods have facilitated epidemiological investigations showing that healthy adult animals can harbour the viral genome. Transmission experiments have demonstrated the pathogenicity of the virus, and indicated that a single species is probably responsible for all the infections observed. The virus isolated from infected *C. gigas* larvae has been formally classified as a member of the *Herpesviridae* under the name ostreid herpesvirus 1 (OsHV-1). Characterization of the genome yielded an estimated size of 207 kbp. The capsid morphology and genome sequence of OsHV-1 have been studied in order to assess its phylogenetic status in relation to vertebrate herpes viruses. The conserved gene that comes closest to being herpes virus-specific encodes the putative ATPase subunit of the terminase, an enzyme complex involved in packaging viral DNA into preformed capsids. Moreover, similarities between the two groups in capsid structure and mechanisms of

capsid maturation tip the balance of evidence in favour of a common origin.

#### OYSTER HERPES VIRUS 1 (OSHV-1) INDUCED GENES IN THE PACIFIC OYSTER (*CRASSOSTREA GIGAS*). T. Renault, N. Faury, V. Barbosa-Solomieu, K. Moreau, C. Brunetière, D. Saulnier and J.-F. Pepin. IFREMER, La Tremblade, France.

Since 1972, several herpes-like virus infections have been reported among different bivalve species around the world. Viral detection was often associated with high mortality rates in spat and larvae of bivalves, including Pacific oyster (*Crassostrea gigas*). The genome characterization of the virus infecting *C. gigas* allowed it to be included in the *Herpesviridae* family as an unassigned member and named Oyster Herpes virus 1 (OsHV-1). The specific objective of the present study was to search for OsHV-1-induced genes expressed in adult *C. gigas* oysters. The suppression subtractive hybridisation (SSH) technique has been used. Oyster haemocytes in contact with infected and non-infected ground larvae were used to extract RNA. Briefly, cDNA was obtained from 1 µg of each hemocyte RNA (control and after virus contact) using the SMART PCR cDNA Synthesis Kit (Clontech), which allowed the full-length amplification of cDNA from mRNA transcripts. A SSH assay was then performed using the PCR-Select cDNA Subtraction Kit (Clontech). The forward-subtracted sample (genes present or up-regulated in presence of the virus) was then used in PCR to amplify the differentially expressed sequences. Cloned PCR products were sequenced using a 3100 Avant Genetic analyser ABI prism (Applied Biosystem). BlastX analysis of the sequenced differentially regulated clones resulted in unique homologues being identified of which some have never been reported previously in any bivalve species. One of the promising candidates corresponds to a homolog with glypican 6 precursor genes. Glypican 6 is a cell surface proteoglycan bearing heparan sulfate. Heparan sulfate proteoglycans serve as cellular receptors for herpes viruses.

#### ECOLOGICAL SERVICES RENDERED BY CULTURED EASTERN OYSTERS. Robert B. Rheault, Moonstone Oysters, 1121 Mooresfield Rd., Wakefield RI 02879.

It has been shown that wild and cultured oysters provide many ecological services that benefit both the environment and wild oyster stocks. (Shumway et al. 2003, Newell 2004, 2002) The services rendered by commercial oyster aquaculture include: removal of nutrients (both by harvest and enhanced bacterial denitrification); improving water clarity and water quality by enhance-

ing sedimentation; adding larvae to the wild; sequestering tons of carbon and creating a structurally diverse habitat for other marine organisms.

When an oyster is harvested approximately 0.52 g N and 0.16 g P are physically and permanently removed from the marine environment. The combined harvest of approximately 700 million cultured Eastern Oysters removes 357 metric tons of nitrogen and 110 metric tons of phosphorus from the marine environment annually. Additional nitrogen is removed by denitrification associated with these farms. These oysters sequestered 51,559 metric tons of carbon in their shell and released an estimated  $1.7 \times 10^{15}$  larvae into the wild. Cultured oysters clear an estimated 94 million cu meters of water daily, while thousands of acres of oyster culture grounds provide a habitat for billions of diverse marine organisms.

These factors were considered in light of the recent effort to list the Eastern oyster as an endangered species. Unavoidable damage to the oyster culture industry would have severe negative impacts on wild populations and coastal water quality as well.

**ASSESSMENT OF THREE STAINS INTERNALLY MARKING MOLLUSC SHELLS OF *CONCHOLEPAS CONCHOLEPAS* AND *MESODESMA DONACIUM*.** José M. Riascos V.<sup>1</sup>, Nury Guzmán<sup>2</sup>, Jürgen Laudien<sup>3</sup>, Olaf Heilmayer<sup>3</sup> and Marcelo Oliva<sup>1</sup>. <sup>1</sup>Universidad de Antofagasta, Investigaciones Oceanológicas, <sup>2</sup>Institut de Recherche pour le Développement, Bondy Cedex, FR, <sup>3</sup>Alfred-Wegener Institute for Polar and Marine Research.

Different stains have been used to internally mark the calcified structures of mollusc shells and thereafter for the validation of growth estimates. As an interspecific variation in marking success is known, an assessment of suitability is necessary. The potential of Calcein, Alizarin red and Strontium-chloride-hexahydrate (SCH) was investigated for the gastropod, *Concholepas concholepas*, and the surfclam, *Mesodesma donacium*, two molluscs of commercial importance in Chile. Wild specimens collected off Antofagasta, Northern Chile, were marked using two concentrations of each stain and two immersion periods as well as a control. After immersion, animals were reared for 20 days to allow growth. Mortality, body condition and growth were measured to assess the effects of treatments. For the detection of the incorporated marks, individuals were culled and shell sections analyzed under a fluorescence microscope (Calcein, Alizarin) and a scanning electron microscope (SCH), respectively. The treatments did not show significant effects on mortality and body condition. However, the growth rate of *C. concholepas* was significantly different ( $P < 0.05$ ) among stains (SCH < Alizarin < Calcein). Calcein markings were successful in all treatments (50 and 100 mg l<sup>-1</sup>, 3 and 6 hrs.) whereas Alizarin only yielded comparable results with 100 mg l<sup>-1</sup> and 6 hrs. SCH just produced faint marks, even at high concentrations. Markings in *C. concholepas* were clearer compared to *M.*

*donacium*, which can be attributed to the gastropod's fast growth rate (0.43  $\mu\text{m d}^{-1}$ ). Calcein was assumed to be the best marker and did not considerably affect the viability of the species studied.

**INTERACTIONS BETWEEN THE SURFCLAM (*MESODESMA DONACIUM*) AND THE COMMENSAL POLYCHAETA (*POLYDORA BIOCCIPITALIS*) IN NORTHERN CHILE.** José M. Riascos V.<sup>1</sup>, Olaf Heilmayer<sup>2</sup>, Marcelo Oliva<sup>3</sup> and Jürgen Laudien<sup>2</sup>. <sup>1</sup>Universidad de Antofagasta, Investigaciones Oceanológicas, <sup>2</sup>Alfred-Wegener Institute for Polar and Marine Research, <sup>3</sup>Universidad de Antofagasta, Investigaciones Oceanológicas.

Although the surfclam, *Mesodesma donacium*, is one of the most important species for shellfisheries in Chile and the infestation by the symbiotic polychaete, *Polydora bioccipitalis*, reaches 31.4%, nothing is known about this species association. Monthly samples from Hornitos (Northern Chile) were collected to determine the shell length (anterior-posterior) when commensalism first occurs, and its effect on the body condition index (BCI = dry tissue mass/internal cavity volume  $\times 100$ ). The temporal pattern of the symbiosis was studied by measuring the thickness of the blisters walls formed in the bivalve shell as an estimation of duration of the association. In addition, a laboratory experiment was performed to assess if the commensal relationship affects the growth rate of the host. Preliminary results indicate that the symbiosis can only be found in clams larger than 33.3 mm. As *M. donacium* undergoes a migration from the intertidal (juveniles) to the lower subtidal (post-juveniles and adults) during its life cycle, it appears that the symbiosis only occurs during a later ontogenetic stage when individuals inhabit the subtidal level. BCI was significantly different ( $P < 0.05$ ) between hosting and non-hosting clams. Although the internal shell cavity was smaller in hosting clams, thus affecting considerably the BCI, the proportion of dry tissue mass was not significantly different. This suggests that *M. donacium* is well adapted to the commensal. Thickness of blister walls showed differences (t-test,  $P < 0.05$ ) between months: thinner blister walls were found in May, June and July, suggesting a seasonal pattern of the commensalism.

**GENETIC STRUCTURE OF OYSTER REEFS ALONG THE ALABAMA GULF COAST.** Gillian Richard and Charles Brockhouse. University of South Alabama, 307 N. University Blvd., LSCB Rm 124, Mobile, AL 36688.

The oyster bed productivity in areas, such as Mobile Bay, has abruptly declined in recent years despite the absence of fishing pressures. These declines have negatively affected the commercial

and recreational fisheries in Alabama and may have also influenced the Bay's natural ecosystem in unknown ways. In an attempt to increase oyster stocks in Mobile Bay, a restoration program has been initiated. To effectively manage an oyster reef restoration project an understanding of the genetic structure of the oyster population(s) and hence, the extent of local larval transport and genetic exchange among the oyster reefs in the natural environment is of considerable practical importance. This study has established a genetic map of the region by investigating the genetic variation and differentiation among five oyster reefs spanning the Alabama gulf coast. The results indicated high levels of genetic diversity within sampled reefs and  $F_{ST}$  revealed little genetic differentiation among the samples. An estimator of gene flow indicated that the main commercial oyster reef, Cedar Point Reef, is the primary source of recruits for the area.

#### CFD ANALYSIS OF SHELLFISH AQUACULTURE GEAR USED IN INTER-TIDAL AND SUB-TIDAL LOCATIONS.

**John Richardson<sup>1</sup>, Carter Newell<sup>2</sup>, Dror Angel<sup>3</sup>, Tessa Getchis<sup>4</sup>, Andy Suhrbier<sup>5</sup>, Jonathan Davis<sup>6</sup> and Daniel Cheney<sup>7</sup>.** <sup>1</sup>Blue Hill Hydraulics Incorporated, <sup>2</sup>Great Eastern Mussel Farms, <sup>3</sup>Massachusetts Institute of Technology, <sup>4</sup>Connecticut Sea Grant Extension Program, <sup>5</sup>Pacific Shellfish Institute, <sup>6</sup>Taylor Resources Baywater, Inc., <sup>7</sup>Pacific Shellfish Institute.

An environmental and technical assessment of alternative methods used to cultivate bivalve shellfish was completed as part of the National Marine Aquaculture Initiative (NMAI). Included in the list of study sites were locations in Washington State and Connecticut where aquaculture is being carried out in inter-tidal and sub-tidal waters using bag-on-bottom, bag-on-rack, net-protected or cage culture methods. To evaluate the effectiveness of these methods used for shellfish aquaculture and to better understand their effect on the local environment, experimental and numerical studies of culture unit hydrodynamics were completed. These studies involved the acquisition of near-field velocity measurements, dye studies, and construction of three-dimensional numerical models based on computational fluid dynamics (CFD) techniques. Of particular interest are the use of fractional volume techniques to model nets and bags, and the use of a kinetics algorithm to calculate phytoplankton consumption within the culture units. These techniques allow the practitioner to accurately simulate three-dimensional flows through the different types of culture units and to estimate food availability within the culture units. The results of the modeling were used to help provide explanation of the field data sets, to identify "holes" in the data sets, and to compare the performance of the different shellfish aquaculture practices. The methods developed for this study can also be used as an aid to evaluate siting concerns and to customize gear designed to work optimally at specific locations.

#### LINKAGES BETWEEN CELLULAR BIOMARKER RESPONSES AND REPRODUCTIVE SUCCESS.

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An important issue for cellular biomarker assessments is whether they are related to higher level responses (e.g. are there linkages between cellular and organismal responses that could translate into population level effects?). The purpose of these studies was to evaluate if there were relationships between lysosomal destabilization or glutathione concentrations and gamete viability of oysters, *Crassostrea virginica*. Oysters were collected from reference and polluted field sites during the peak spawning period (May-June). Hepatopancreas (e.g. digestive gland) tissues were dissected and a portion was used immediately for lysosomal destabilization assays (based on neutral red retention), and glutathione concentrations were also determined for both hepatopancreas and gonadal tissues. Eggs and sperm from the same adults were also used to conduct embryo development assays with reference seawater collected from a clean site, site water, and also a range of Cd concentrations (the Cd exposures were used to determine if there were differences in susceptibility to pollutants). Baseline embryo development success (e.g. percent normal development when the assays were conducted with reference seawater or site water) was related to lysosomal destabilization, but not glutathione status. However, the susceptibility of embryos to metal exposures was related to glutathione status, i.e. sensitivity to Cd exposures increased with decreasing glutathione levels. These studies support the hypotheses that there are linkages between these cellular biomarker responses and embryo development success and susceptibility to pollutant stress. These kinds of effects on reproductive success could lead to subtle but significant long-term effects on recruitment and viability of oyster populations.

#### OXYTETRACYCLINE TREATMENT OF WITHERING SYNDROME IN RED ABALONE (*HALIOTIS RUFESCENS*).

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Since the mid 1980's the disease withering syndrome (WS) has severely impacted wild and cultured California abalone (*Haliotis* spp.). The causative agent of WS "*Candidatus Xenohaliotis californiensis*" infects gastrointestinal epithelia. The broad-spectrum antibiotic oxytetracycline (OTC) has been shown to control infec-

tions in cultured and broodstock abalone as a therapeutic in medicated feed. We examined the efficacy and pharmacokinetics of OTC in infected red abalone, *Haliotis rufescens*, medicated with an artificial diet containing 1.85% active OTC at a rate of 103.4 mg/kg abalone for 10, 20 or 30 days at 13.4°C. The medication was 97% effective in clearing RLP infections in the 10 day treatment and 100% in the 20 and 30 day treatments. Significant differences were observed in prevalence ( $p < 0.0001$ ) and intensity of infection in post-esophagus ( $p < 0.0001$ ) and digestive gland ( $p < 0.05$ ) of treated abalone compared to untreated abalone. Survival was not appreciably different among treatments due to a thermal refuge from disease expression at low temperature; however during a sequential study at an elevated temperature of 17.3°C, unmedicated abalone suffered higher losses and developed clinical WS at a higher rate than medicated animals. Depletion dynamics in abalone tissues showed animals medicated for 10 days accumulated significantly less OTC than those medicated for 20 or 30 days with significantly lower OTC levels present in foot muscle samples relative to digestive gland samples ( $P < 0.001$ ). In contrast, at 17.3°C abalone only accumulated half as much OTC as those at 13.4°C suggesting that both temperature and duration of medication are important regulators of OTC pharmacokinetics in abalone.

**OVERVIEW AND APPLICATION OF BAY SCALLOP GENOMIC RESOURCES.** Steven Roberts, Marine Biological Laboratory, 7 MBL Street, Woods Hole, MA 02543.

The bay scallop (*Argopecten irradians*) is a commercially important bivalve mollusc that supports culturally significant recreational fisheries along the East Coast of the United States. The bay scallop is also an excellent scientific model to study visual signal transduction, early development, and muscle physiology. Furthermore, like other bivalves, scallops are essential components in maintaining sustainable marine environments. In order to provide basic molecular tools to study scallop biology several approaches have been taken including differential display, degenerative primer based RT-PCR and small-scale expressed sequence tag projects. Several gene products from these combined efforts have been further characterized using comparative analysis of gene expression, *in silico* procedures, and recombinant protein systems. Expressed sequence tag derived genetic markers have been developed and used to evaluate stock enhancement. An overview of the molecular techniques employed will be presented including suggestions on how to develop similar efforts. Experimental results will be presented on transcripts associated with scallop early development and growth. This research was supported by the United States Department of Agriculture (grant #2003-35206-12834) and the County of Barnstable Massachusetts.

**CHARACTERIZATION OF DIFFERENTIALLY EXPRESSED GENES FROM QPX: INSIGHT INTO POSSIBLE VIRULENCE MECHANISMS.** Steven Roberts, Roxanna Smolowitz and Christina Romano, Marine Biological Laboratory, 7 MBL Street, Woods Hole, MA 02543.

Quahog Parasite Unknown (QPX) is a protist, genetically identified as belonging to the family Thraustochytridae in the phylum Labyrinthulomycota (slime mold). It proliferates by endospore formation and has been identified as a significant cause of hard clam mortality in the Northeast United States. QPX causes significant disease and mortality in both cultured and wild clam populations in the region. In order to begin to understand what genes are present in the extracellular proteins of QPX mucus and are associated with QPX virulence, we have started to characterize genes that are expressed in QPX under different conditions. In one experiment focused on temperature influences on gene regulation, QPX cultures were grown at 10°C and 21°C for 10 days. Following incubations, total RNA was extracted from cultures and several differentially expressed genes were identified using a differential display polymerase chain reaction technique (GeneFishing DEG System—Seegene). To our knowledge, these are all novel gene homologs in QPX as only rDNA sequences have been previously characterized. One of the genes upregulated at 10°C is a potassium channel protein, based on DNA sequence homology. Similar proteins have been shown to be important for other pathogens as they can inhibit phagocytosis. Additional transcripts and comparative gene expression analysis in QPX strains under different environmental conditions will be presented. The long term goal of this research is to provide resource managers with basic information on QPX biology that could be used to facilitate hard clam aquaculture and protect wild populations.

**IMPLICATIONS OF HIGH-RESOLUTION GEOPHYSICAL TECHNIQUES IN OYSTER HABITAT IDENTIFICATION: CAPE FEAR RIVER, NORTH CAROLINA.** Kassy Rodriguez, Nancy Grindlay, Lewis Abrams, Troy Alphin and Steven Artabane, University of North Carolina Wilmington.

Eastern oysters (*Crassostrea virginica*) are filter feeding organisms that settle in dense aggregates and positively impact water quality by providing a series of ecosystem functions, which include habitat for other organisms, filtration, and habitat stability. The United States Army Corps of Engineers created dredge spoil islands throughout the lower 20 km of North Carolina's Cape Fear River as a result of channel dredging. These activities have altered water flow and increased siltation, reducing the amount of hard substrate available for oyster spat settlement and growth. Currently, small amounts of hard substrate remain in the lower river



and routinely experience high overspat events, followed by high mortality from a combination of competition and high sedimentation. Sidescan sonar and sub-bottom profiling systems were used to map four areas within a 15 km long section of the lower Cape Fear River to locate living and buried oyster reefs. The southernmost area contains living oysters, while the northernmost areas are along dredge spoil islands and are devoid of living populations, despite the presence of Indian middens, which may suggest a localized historic occurrence of oysters and the potential for buried oyster reefs. Potential buried reef locations have been identified for ground-verification. Hypothesizing that oysters should be able to grow where they once thrived, these data will be used to guide placement of oyster cultch. If these sites prove suitable for new oyster cultivation, this geophysical technique has important implications for the success of future oyster restoration projects in southeastern North Carolina.

**RECRUITMENT AND SUCCESSION VARIABILITY IN OYSTER FOULING COMMUNITIES IN BAHIA SAN QUINTIN, BAJA CALIFORNIA, MEXICO.** Laura F. Rodriguez, University of California, Davis, Section of Evolution and Ecology, Davis, CA 95616.

Bahia San Quintin is a shallow bay on the Pacific coast of the Baja California peninsula, Mexico. Historically dominated by soft sediment systems, the bay currently supports a locally important oyster aquaculture industry. In this system, oysters (*Crassostrea gigas*) are cultivated on ropes and remain suspended in the water column for about one year, until they attain market size. These systems provide a significant amount of available substrate for the development of diverse fouling communities, which are dominated by ascidians, sponges, bryozoans, and hydrozoans. These fouling communities harbor non-indigenous species, one of which is the Australian ascidian, *Microcosmus squamiger*. This ascidian can attain pest-like densities, completely covering oyster ropes and likely limiting the amount of food and dissolved oxygen accessible to the oysters. Oyster farmers view fouling communities as a nuisance that both harm their oyster crop and increase their handling time in cleaning and packaging oysters. To investigate the impact of these fouling communities on the oyster crop I have been taking monthly recruitment and community succession samples at three sites within Bahia San Quintin for the past two years (2004–2005). I used oyster shells as substrate panels to look at the abundance and prevalence of fouling organisms after 1, 2, 3, 6, and 12 month soaking periods. Results from this study establish recruitment patterns and variability, and community development and succession trends following recruitment. These results, coupled with seasonal and water temperature information, will help develop management options to mitigate impacts of detrimental fouling species.

**OCEAN TEMPERATURE, FOOD ABUNDANCE AND DISEASE: CONTRIBUTORS TO REPRODUCTIVE FAILURE IN RED ABALONE.** Laura K. Rogers<sup>1,2</sup>, Richard F. Dondanville<sup>3</sup>, L. Ignacio Vilchis<sup>4</sup>, Mia J. Tegner<sup>4,\*</sup>, Beverly Braid<sup>2</sup>, James D. Moore<sup>1,2</sup>, and Paul K. Dayton<sup>4</sup>. <sup>1</sup>California Department of Fish and Game, <sup>2</sup>U.C. Bodega Marine Laboratory, 2099 Westside Rd., Bodega Bay, CA 94923, <sup>3</sup>5342 Winding View Trail, Santa Rosa, CA 95404, <sup>4</sup>Scripps Institution of Oceanography, University of California San Diego, La Jolla, CA 92093, <sup>\*</sup>Deceased.

Changes in ocean climate can have dramatic, non-linear impacts on the population dynamics of marine invertebrate populations. We investigated the impacts of water temperature and other factors on reproduction in red abalone (*Haliotis rufescens*) (1) from the wild, (2) from laboratory experiments varying water temperature and kelp rations, and (3) disease exposure. We examined body morphology, gonad and digestive gland indices, and histology sections of gonad tissue to determine fecundity. All wild abalone had mature gametes and females averaged more than 2 million mature eggs. Males had total reproductive failure, with no sperm in their testes, at temperatures of 16°C and warmer suggesting a fixed upper thermal limit. Females continued to produce mature eggs at warm temperatures, but were sensitive to reductions in food quantity. No gametes were found in diseased abalone of either sex once their foot muscle started to shrink from disease. Diseased abalone could be distinguished from starved abalone by their abnormal gonad structure. Starved abalone had normal gonad structure, with some gametes, but dramatically reduced gonad (25% of wild) and digestive gland (45% of wild) indices. The temperature-mediated effects on reproduction of individual red abalone described here, combined with temperature's known impacts on abalone growth, kelp abundance and expression of disease, clearly demonstrate temperature's population level consequences. We suggest that temperature be explicitly incorporated into red abalone recovery and management planning since water temperature plays a large role in male fecundity and since ocean temperatures are predicted to rise in the future.

**OYSTER RESTORATION IN AN URBAN LANDSCAPE: CHARACTERIZATION OF A BASIN-WIDE OYSTER POPULATION IN THE LYNNHAVEN RIVER, VIRGINIA.** P. G. Ross<sup>1</sup>, M. W. Luckenbach<sup>1</sup>, A. J. Birch<sup>1</sup> and L. D. Coen<sup>2</sup>. <sup>1</sup>VA Institute of Marine Science, College of William & Mary P.O. Box 350, Wachapreague, VA 23480, <sup>2</sup>Marine Resources Research Institute, South Carolina Department of Natural Resources.

Historically in the Chesapeake Bay, quantitative evaluations of oyster reef restoration have focused on subtidal reefs utilizing fisheries-based metrics (e.g. abundance of market-sized, ~76 mm, oysters). Monitoring is typically initiated after restoration activities have been implemented, with few instances of coordinated data collection before, during and after restoration activities. Beginning in 2006, a large-scale oyster restoration effort, including habitat



and brood stock enhancement, is planned for the Lynnhaven River Basin, a small and relatively closed tidal sub-tributary of the lower Chesapeake Bay. In contrast to most other restoration sites in this region, the Lynnhaven Basin is heavily urbanized with extensive portions of the shoreline covered with stabilization structures such as concrete, wood or metal bulkheads and granite or concrete rubble that can be considered “non-traditional” habitats, which support substantial oyster densities. This type of habitat poses significant challenges for determining initial oyster population size within the basin and basin-level effects of subsequent restoration efforts. We report on characterization of the basin-wide oyster population (overall abundance and size distribution) prior to the fore mentioned restoration activities, including data collection techniques (e.g. sub-meter accuracy surveying Global Positioning Systems [GPS], integrated with an ArcView-based Geographic Information System [GIS]), and challenges regarding extrapolating oyster data to such a large region. Additionally, we describe the extent and relative proportion of varied habitats in this system along with habitat-specific oyster population parameters.

**ROTATIONAL AREA MANAGEMENT IN THE NORTH-WEST ATLANTIC SEA SCALLOP FISHERY: ARE CURRENT MANAGEMENT STRATEGIES FLEXIBLE ENOUGH?** David B. Rudders, William D. DuPaul and Noëlle Yochum, Virginia Institute of Marine Science, Gloucester Point, VA 23062.

Limiting fishing activities in certain areas has gained support as a method to conserve and enhance marine resources. Amendment #10 to the Sea Scallop Fishery Management Plan formally established area rotation as a regulatory strategy to protect aggregations of pre-recruit scallops to optimize gains in yield-per-recruit. The criterion for establishing and re-opening rotational closed areas is dependent upon estimates of the year-over-year growth expressed as a percentage increase of scallop biomass. This strategy is advantageous when large recruitment events are episodic in nature, resulting in a predominant year class. It is also effective because, historically, sea scallop recruitment has been highly variable. Recently, regular recruitment has been observed in the mid-Atlantic region, including the Elephant Trunk Closed Area (ETCA), which was established in July 2004 to protect a large 2002 year class. To evaluate this trend, a fixed grid survey was conducted in October 2005 using both a commercial and a NMFS survey dredge. Results indicate that while the 2002 year class that prompted the closure is present, additional recruitment events have taken place. The addition of these recruits complicates the use of biomass thresholds as triggers for closing and opening rotational management areas. The ETCA is scheduled for a limited opening in 2007. Managers have expressed concern that yield-per-recruit objectives will not be met and there is potential for discard related fishing mortality. We examined the flexibility of the criterion for rotational area man-

agement and various spatial and temporal harvesting strategies to optimize fishery yields and minimize non-harvest mortality.

**THE EFFECT OF CONDITIONING HATCHERY-REARED GREEN SEA URCHINS TO LOW SALINITY ON SURVIVAL AND GROWTH IN THE FIELD.** Michael P. Russell, Villanova University, Villanova, PA 19085.

Harvest data show a precipitous decline in the green sea urchin fishery (*Strongylocentrotus droebachiensis*) in the Gulf of Maine. This situation has prompted studies aimed at enhancing standing stocks by seeding formerly productive fishing grounds with hatchery-reared juveniles. One complicating factor is that these environments are subject to fluctuating salinity. Echinoderms are osmoconformers and generally do not tolerate brackish water. However, green urchins are exceptional in their abilities to acclimate to periodic exposures to low salinity. We conducted a field study to assess the efficacy of conditioning hatchery-reared juveniles to hyposaline conditions prior to releasing them at sites where low-salinity conditions occur. Sea urchins were raised from larvae in the lab and grown from settlement for 15 weeks. This cohort was divided into two groups of equal number and size distribution, and randomly assigned to control and treatment groups. The treatment was subjected to five bouts of low salinity (21‰) for 36 hours every two weeks. As expected, at the end of the acclimation period, the treatment group was significantly smaller than the control. A 2 × 2 factorial ANOVA design was used to establish these samples in a field experiment with site and treatment level as factors in a sea urchin lease-site in the Piscataqua River, New Hampshire. Growth and survival were monitored for one year and the results show no difference in survival, but showed a significant effect of conditioning on growth. Future efforts to establish seeding programs using hatchery-reared juveniles would benefit from incorporating salinity-conditioning prior to release.

**IMPROVED CULTURE TECHNIQUE FOR EDIBLE SEA URCHINS, *STRONGYLOCENTROTUS INTERMEDIUS* LARVAE.** Yuichi Sakai<sup>1</sup>, Yasuko Konda<sup>1</sup>, Saburoh Yanagisawa<sup>2</sup>, Hiroyuki Abe<sup>2</sup>, Tsutomu Nishimura<sup>2</sup>, Mitsutake Omoteya<sup>2</sup> and Makoto Azumi<sup>2</sup>. <sup>1</sup>Hokkaido Institution of Mariculture, 539-112 Honbetsu, Shikabe, Hokkaido, 041-1404, Japan, <sup>2</sup>Hokkaido Aquaculture Promotion Corporation, 4th floor, Hokkaido Dai-2 Suisan Bldg., Kita-3, Nishi-7, Chuo-Ku, Sapporo, Hokkaido, 060-0003, Japan.

The artificial mass production technique for *Strongylocentrotus intermedius* developed in 1985 allowed the production of 6.1 million juveniles in 2002, in the region of Hokkaido, Japan. However, many problems still persist particularly during the larval stage, such as mass mortality (39–100%) as measured the sinking of larvae in early 6 and 8 arms stages and the precarious settlement rate (39–98%). Other problem is the necessity for daily cleaning of

the larval rearing tank. To reduce these problems, increase in the aeration volume and food availability by temporal stop of water flow (8 hours) was attempted. To study the settlement rate, a new criterion was developed to judge the timing of larvae settlement. With the improved technique, the survival and settlement rate increased 1.7 times and 1.1 times, respectively. With this, the total amount of larval food (*Chaetoceros gracilis*) reduced and the daily cleaning of the rearing tank could be stopped, thus, reducing the load in hatchery operation. The cost of juvenile production reduced by about 10%.

**POSSIBLE WAYS TO PREVENT *CRASSOSTREA GIGAS* OYSTER SUMMER MORTALITY IN FRANCE.** Jean François Samain, Pierre Boudry, Lionel Degremont, Patrick Soletchnik, Michel Ropert, Edouard Bédier, Joseph Mazurié, Jean L. Martin, Jeanne Moal, Michel Mathieu, Stéphane Ponvreaux, Christophe Lambert, Jean M. Escoubas, Jean L. Nicolas, Frédérique Le Roux, Tristan Renault, Thierry Burgeot and Cédric Bacher. Ifremer, Brest Center, BP 70, 29280 Plouzané, France.

A first understanding of the complex interactions between oyster, environment and pathogens was obtained during the MOREST (2001–2005) project on summer mortality events of *Crassostrea gigas* oysters in France. High trophic conditions control reproduction intensity and the susceptibility to bacterial infection. However the risk begins when temperatures exceed 19°C, mainly when an additional stressor, proximity to sediment is present. This varied between years and runoff from watersheds is implicated. Finally, a genetic resistance exists among some natural oyster populations. None of these different factors can separately account for summer mortality, and all of these conditions seem necessary to reproduce the event.

In the present case study of *C. gigas* oyster summer mortality, these necessary interactions provide different possibilities to prevent or reduce the problem by breaking one of them. Short term and long term options of reducing risk will be presented and discussed. Short term options include culture practices (temperature, trophic conditions, sediment distance, density, rearing steps, reduction of reproduction and stress). Options can depend also on biological material, with possibility of using triploids. Long term options by genetic selection of resistant oysters or by management of nutrients and organic matter from river watersheds are also possibilities. A risk analysis is necessary for each growing area to adapt the best strategy to the local environmental and culture conditions. The assessment of the economical aspects will be necessary before validation of these choices. This case study presents synergistic activities of scientists and resource managers in enhancing or restoring oyster populations in France.

**A HISTOLOGICAL INVESTIGATION OF OYSTER PARASITES AND PATHOLOGY IN CHINA.** Emily Scarpa, Susan Ford, Ximing Guo, Lisa Ragone Calvo and David Bushek. Haskin Shellfish Research Laboratory, Rutgers, The State University of New Jersey, 6959 Miller Avenue, Port Norris, New Jersey 08349 USA.

The proposed introduction of *Crassostrea ariakensis* into Chesapeake Bay as a means to restore oyster populations presents a number of potential risks, such as pathogens and pathological conditions that require careful examination and research before approval. Parasites occurring, even at low prevalence, in *C. ariakensis* within its native distribution may seriously impact this or other species in a different environment. Pathogens present in oyster species coexisting with *C. ariakensis* in its native habitat may also present problems if they are able to use *C. ariakensis* as a host or reservoir. In this survey, 27 samples were collected from eight provinces along China's coastline. Cross-sections of individual oysters were preserved in Davidson's fixative. Genetic analysis of ethanol preserved gill samples was conducted to identify species of individual oysters. Nine samples were selected based on presence of *C. ariakensis*. These oysters, representing six sites and consisting of *C. ariakensis* and a number of coexisting species, were processed by normal histological procedures and examined. Individual *C. ariakensis* were also examined using a fluorescence immunostaining technique to identify presence of *Perkinsus* sp. infections. Observed parasites included ciliates, such as *Sphenophrya*-like ciliates and trichodinids; crustaceans, including intestinal copepods; coccidians, including *Nematopsis* and a coccidian-like organism; rickettsia/chlamydia-like organisms; and trematodes. Overall prevalence of any particular parasite did not reach above 3% and averaged less than 1%. However, prevalences of coccidian-like and *Nematopsis* species were as high as 45% and 64%, respectively, at specific sites. To date, no evidence of a significant pathological impact has been observed.

**AN INVESTIGATION OF CILIATE XENOMAS IN *CRASSOSTREA VIRGINICA*.** Emily Scarpa<sup>1</sup>, Susan Ford<sup>1</sup>, Bruce Smith<sup>2</sup> and David Bushek<sup>1</sup>. <sup>1</sup>Haskin Shellfish Research Laboratory, Rutgers, The State University of New Jersey, 6959 Miller Avenue, Port Norris, NJ 08349 USA. <sup>2</sup>New Hampshire Fish & Game, Durham, NH 03824.

Since the late 1990s, unusually high prevalences of xenomas have been noted during routine histological examination of oysters from Great Bay, New Hampshire. Xenomas are formed when intracellular parasites accumulate within host cells, causing them to hypertrophy. Although in fish xenomas are commonly caused by microsporidians, in oysters xenomas are caused by ciliates, genus *Sphenophrya*, and are rare. Because they are macroscopically visible on gills, the marketability of infected oysters has been questioned. In this study, samples were collected every fall from 1997 through 2005 and processed using normal histological procedures.

In 2005, counts were also made of macroscopically visible xenomas. Prevalence varied according to site within Great Bay and also by year. In histological sections, it has increased notably since 1997, when only 1% of oysters were affected. In 2004, prevalence ranged from 33% to 82% between sites. Densities were mostly below 20 xenomas per histological section, but reached as high as 173. Macroscopically, samples from 2005 contained a mean 15.5 xenomas per oyster, ranging from zero to more than 100. The xenomas were located in gill water tubes and were often large enough to occupy the entire cross sectional area. They cause localized epithelial erosion and most likely impede water flow. Nevertheless, the histological appearance of the remaining tissues was not obviously affected and there was no clear correlation between oyster size and infection. There was an inverse relationship between the prevalence of *Haplosporidium nelsoni* (MSX) infections and that of xenomas, although the reason is presently unclear.

**GONAD PRODUCTIVITY, COLOR AND TEXTURE IN *STRONGYLOCENTROTUS FRANCISCANUS* FED THREE PREPARED DIETS.** Susan C. Schlosser<sup>1</sup>, A. L. Lawrence<sup>2</sup>, S. A. Watts<sup>3</sup>, P. D. Tom<sup>4</sup> and J. M. Lawrence<sup>5</sup>. <sup>1</sup>University of California Sea Grant Program, Eureka, CA. <sup>2</sup>Texas A & M University, Port Hansen, TX. <sup>3</sup>University of Alabama, Birmingham, AL. <sup>4</sup>University of California, Davis. <sup>5</sup>University of South Florida, Orlando, FL.

Commercial sized *Strongylocentrotus franciscanus* (mean size 104.2 mm TD, 434.4 g) were collected from fishing grounds. An initial sample was dissected immediately. The remainder were placed individually in 25 l aquaria (n = 8/treatment) and fed one of three prepared diets. The fourth group was unfed. Dry body compartment indices, daily dry feed intake, gonad color, hardness, and resilience were measured after 60 days. A group of processed, Grade A gonads were compared with laboratory gonads. Results were compared by One Way ANOVA. There were no significant differences for dry gonad and test index, or L (gonad lightness). Dry gut index was significantly greater in prepared Diets 2 and 3 compared to the unfed treatment. Dry lantern index of the unfed group was significantly lower than the initial sample and all prepared diet treatments. Gonad redness (a) was significantly greater in the initial sample than the unfed group. Gonad yellowness (b) was significantly greater in the commercially processed gonads than in prepared diets 1 and 2 and the unfed group. Hardness of commercially processed gonads was greater than Diet 1. Resilience was significantly greater in commercially processed gonads than diets 1 and 3 and the unfed treatment. Feed intake was significantly different with Diet 1 (0.45g/urchin/day) > Diet 2 (0.41) > Diet 3 (0.31). Sea urchin gonads from Diet 3 were overall most similar to processed gonads in color and texture. Diets had similar protein, fiber, and fat content.

**PERKINSUS SPP. AND BONAMIA SPP. INFECTIONS IN *CRASSOSTREA ARIAKENSIS* MAINTAINED IN A FULLY CONTAINED AQUACULTURE SETTING.** Eric J. Schott, José A. F. Robledo, Mohammad R. Alavi, Keiko Saito, Satoshi Tasumi, Wolf T. Pecher and Gerardo R. Vasta. COMB. UMBI, University of Maryland, 701 Pratt St., Suite 236, Baltimore, MD 21202, USA.

In the Chesapeake Bay, diseases caused by *Perkinsus marinus* and *Haplosporidium nelsoni* (Dermo and MSX, respectively) have contributed to drastic declines of populations the native oyster, *Crassostrea virginica*. The Asian oyster, *C. ariakensis*, which grows readily to market size in Dermo-endemic Chesapeake Bay waters, is being considered for introduction to restore oyster populations. While apparently tolerant to *P. marinus*, *C. ariakensis* exposed to Bay waters may reach prevalences of up to 80%, raising the possibility that it could serve as a reservoir in which the parasite could increase its virulence. A crucial question to address is whether *P. marinus* can be transmitted from *C. ariakensis* to naïve *C. virginica*. We conducted cohabitation experiments with *Perkinsus*-infected *C. ariakensis* and *Perkinsus*-free *C. virginica*. The prevalence of *Perkinsus* infection in both 'donor' and potential 'recipient' populations was assessed at 2 and 4 weeks by PCR-based methods. After four weeks, *Perkinsus* was present in *C. virginica*. A potential protozoal disease of *C. ariakensis*, *Bonamia ostraea*, has been associated with mortalities of experimental populations of *C. ariakensis* in Pamlico Sound of NC. The potential exists for *Bonamia* sp. to be present in Chesapeake Bay. We previously detected PCR amplicons indicative of *Bonamia* spp. in *C. ariakensis* reared in the Chesapeake Bay. We are conducting cohabitation studies in which *B. ostraea*-infected *Ostrea edulis* are co-cultured with *C. ariakensis* for four weeks. We are currently in the process of analyzing possible transmission using PCR-based methodologies.

**THE CALIFORNIA BAREFOOT ECOLOGIST PROGRAM: A COOPERATIVE STOCK ASSESSMENT MODEL FOR THE CALIFORNIA RED SEA URCHIN FISHERY.** Steve Schroeter<sup>1</sup>, John Duffy<sup>2</sup> and Peter Halmay<sup>3</sup>. <sup>1</sup>Marine Science Institute, University of California, Santa Barbara, Santa Barbara, CA 93106, <sup>2</sup>California Department of Fish and Game, <sup>3</sup>311103 Hwy 67, Lakeside, CA 92040.

The red sea urchin fishery in California is currently managed by a combination of size limits, seasonal and area closures, and a restricted access program. This resource is largely assessed through fishery dependent measures (i.e. landings summarized in 10 nm × 10 nm blocks and port samples of size distributions of catches) and a small number of projects that have gathered fishery independent data. Recent ecological work indicates that fishery

dependent measures are not up to the task of stock assessment and that work in the fishery independent realm does not adequately take into account the meta-population structure or the extreme spatial patchiness of the resource. This shortcoming is due in large part to the logistical requirements dictated by a resource with extreme spatial patchiness at scales ranging from 10s to 1000s of meters. We discuss a possible solution which involves the design and implementation of a cooperative stock assessment model using fishermen trained as 'Barefoot ecologists' to collect data. We describe how fishermen collect data both on the harvest grounds during routine fishing operations, and using the same sampling design, at sites outside the harvest grounds, thus providing a comprehensive fishery independent assessment of the red sea urchin stocks. We use field data to illustrate the sampling problems and to present two possible solutions we have developed to address them.

**MOLECULAR MARKERS FOR PARENTAGE ASSIGNMENT IN HARD CLAM (*MERCENARIA MERCENARIA*) STOCKS.** Gail P. Scott<sup>1</sup>, Mark D. Camara<sup>2</sup>, Kelly R. Johnson<sup>1</sup>, Ryan B. Carnegie<sup>1</sup>, Standish K. Allen Jr.<sup>1</sup> and Kimberly S. Reece<sup>1</sup>. <sup>1</sup>VIMS, The College of William and Mary, P.O. Box 1346, Gloucester Point, VA 23062, <sup>2</sup>USDA-ARS, OSU-Hatfield Marine Science Station.

The hard clam, *Mercenaria mercenaria*, is currently the most important aquaculture species on the east coast of the United States, yet development of selected clam brood stock is still very much in its infancy. An efficient breeding program that maximizes selection intensity, while maintaining genetic diversity, could result in substantial benefits to the industry. Selective breeding in molluscs, however, is complicated by their high fecundity and high variance in reproductive success, which rapidly can result in inbreeding. Because larval molluscs are impossible to physically tag or mark individually, one of the only practical methods of controlling inbreeding is to rear families separately; however, this requires enormous amounts of labor in both the hatchery and field. In addition, separately reared families must be replicated extensively to randomize environmental effects. Alternatively, using molecular genetic markers, families can be mixed for rearing, desired traits can be measured, and individuals can be retrospectively assigned to families based on multi-locus genotypes. We have recently developed both microsatellite and single nucleotide polymorphism (SNP) markers in *M. mercenaria*. Male and female individuals from hatchery strains originating in different regions were used as parents in controlled laboratory spawns to produce 50 distinct full-sib families. Parental tissue and larvae from each of the 50 crosses were preserved to provide progeny samples with independently known parentage for testing the power of the markers for accuracy at pedigree reconstruction.

**INTRODUCTION OF THE EUROPEAN SEA URCHIN (*PARACENTROTUS LIVIDUS*) IN A LAND BASED INTEGRATED SYSTEM IN ISRAEL.** Muki Shpigel and Ingrid Lupatsch. National Center for Mariculture, Eilat, Israel.

The sea urchin, *Paracentrotus lividus*, was introduced as a candidate species in a fish, seaweed and sea urchin integrated system in Eilat, Israel. While fish represent the main product, effluents from fish culture support the growth of the macroalgae, *Ulva lactuca* and *Gracilaria conferta*. Both seaweeds were used as a biofilter to remove dissolved nutrients from the water and as food for the sea urchins. Sea urchin performances in parameters such as growth, survival, food conversion ratio (FCR), protein and energy use, gonad production, and gonad color were evaluated. Growth from spawning to commercial size (45 mm) on the seaweed diet took ca 36 months, FCR on a wet weight basis ranged between five and seven, and survival rates from settlement were 70–80%. Three months before harvest, prepared diets are introduced in order to improve raw growth.

**POLYCULTURE OF THE SHRIMP *LITOPENAEUS VANNAMEI* AND THE SEA URCHIN *LYTECHINUS VARIEGATUS*.** Anthony J. Siccardi III<sup>1</sup>, Addison L. Lawrence<sup>1</sup> and Stephen A. Watts<sup>2</sup>. <sup>1</sup>Texas A&M University System, Port Aransas, Texas, <sup>2</sup>University of Alabama, Birmingham, Alabama.

Polycultures potential has been researched for many species as it represents a potential cost savings to aquaculturists. This study examines the feasibility of *L. vannamei* and *L. variegatus* polyculture. Ninety-six 19-L tanks connected to a semi-closed recirculating seawater system were stocked with one *L. vannamei*. Each tank contained two elevated mesh cages to which 0, 2, or four sea urchins/tank were added. Sea urchins were fed ca. 0.3 grams of a 27% protein research feed per day per urchin. Treatments (N = 16) were: 1) two urchins per tank plus 0.2 grams per day of a supplemental commercial 45% protein shrimp feed, 2) two urchins per tank with no supplemental shrimp feeding, 3) four urchins per tank with no supplemental shrimp feeding, 4) zero urchins per tank with 0.2 grams per day shrimp feed, 5) zero urchins per tank with 0.4 grams per day shrimp feed and 6) zero urchins per tank and no supplemental shrimp feeding. Weight gain was similar in shrimp who consumed fecal material from 4 sea urchins to those fed 0.2 grams per day shrimp feed ( $P > 0.05$ ). Weight gain was higher in shrimp who consumed fecal material from two urchins plus 0.2 grams per day of supplemental shrimp feed, and was equivalent to shrimp fed 0.4 grams per day shrimp feed ( $P > 0.05$ ). Fecal material alone from two urchins was not able to sustain shrimp growth. Data indicates *L. vannamei* is able to utilize sea urchin fecal material which enhances the likelihood of successful commercial polyculture.

**MOLTING AND CHANGES IN BODY COMPOSITION OF THE PACIFIC WHITE SHRIMP, *LITOPENAEUS VANNAMEI*, DURING STARVATION.** Anthony J. Siccardi III<sup>1</sup>, William H. Neill<sup>2</sup>, Addison L. Lawrence<sup>3</sup>, Delbert M. Gatlin III<sup>2</sup>, Frank L. Castille<sup>1</sup> and Joe M. Fox<sup>4</sup>. <sup>1</sup>Texas A&M University System, Port Aransas, Texas, <sup>2</sup>Texas A&M University, College Station, Texas, <sup>3</sup>Texas A&M University System, Port Aransas, Texas, <sup>4</sup>Texas A&M University, Corpus Christi, Texas.

Starvation studies using the comparative slaughter method contribute to better understanding of animal bioenergetics. To assess compensatory growth, researchers typically starve animals for brief periods. Few studies of shrimp or other crustaceans have assessed changes in body composition upon starvation over periods as long as one month. To determine the effect of long-term starvation, four weight classes (initially weighing 5.51, 7.19, 14.10, and 16.59 g per shrimp) of *L. vannamei* were individually held without feeding in 400 tanks (100 shrimp and tanks per size class). Ten shrimp were removed weekly, weighed and then individually analyzed for energy, ash, moisture and protein until mortality was complete. Shrimp continued to molt throughout the study, despite the energy loss and stress involved in the molting process. The daily loss of protein per shrimp was described by the following equation: protein loss ( $\text{g shrimp}^{-1} \text{ day}^{-1}$ ) =  $0.0045 * W(g)^{0.92}$ , where  $W$  = geometric mean of body weight between the initial and final weights after 28 days of starvation. Daily loss of energy was described as energy loss ( $\text{cal shrimp}^{-1} \text{ day}^{-1}$ ) =  $37.15 * W(g)^{0.87}$ . Molting reduced the volume of the starved 5.51 g shrimp by approximately 34%. A reduction in volume also was noted for *L. vannamei* fed below their maintenance protein and energy requirements. The authors believe this loss of volume reflects a tendency toward homeostatic maintenance of tissue water and energy density requisite for functional integrity.

**LONG-TERM STUDY OF FEED INTAKE AND GROWTH UNDER CONSTANT TEMPERATURE REGIME IN THE GREEN SEA URCHIN (*STRONGYLOCENTROTUS DROBACHIENSIS*).** Sten I. Siikavuopio, Trine Dale and Bjørnsteinar Sæther, Norwegian Institute of Fisheries and Aquaculture Research, Fiskeriforskning, 9291 Tromsø, Norway.

A long-term study of feed intake, somatic growth and gonad growth of green sea urchin, *Strongylocentrotus droebachiensis* was conducted. Three size groups of sea urchins (initial diameter: 35 mm (S), 45 mm (M) and 50 mm (L)) were held individually in square chambers for 850 days and fed *ad libitum* a formulated moist feed. The animals were kept at constant water temperature (10°C) and under simulated natural photoperiod regime. Feed intake (FI), feed conversion efficiency (FCE), somatic growth, gonad index (%) and gonad colour were measured. The feed intake in all size groups seemed related season, as the feed intake was significantly higher in summer compared to winter. The multiple linear regression show that the somatic growth curves of different

size groups had significantly different slopes ( $P < 0.05$ ) ( $S = 37.472 + 0.025x$ ,  $M = 44.447 + 0.019x$ ,  $L = 52.286 + 0.01x$ , where  $x$  = days). S group had the fastest increase in test growth ( $P < 0.05$ ), followed by size group M, and L. There were no significant differences in gonad index, FCR and gonad colour between the groups at the end of the experiment. Overall, our finding suggest that the feeding rates of the adult green sea urchins show a strong correlation with season, possibly due to the annual reproduction cycle.

**OSCILLATIONS IN SEA URCHIN POPULATIONS ALONG THE NORWEGIAN COAST.** Knut Sivertsen, Finnmark University College, Alta, Norway.

Barren grounds appeared from kelp beds along the Norwegian coast caused by heavy grazing by the sea urchins (*Strongylocentrotus droebachiensis*) during the 1970s and 1980s. In the 1990s kelp re-established in the southern part after the sea urchin populations had declined. No increase of predators or no parasite infection in the sea urchins was registered in the re-established areas. A hypothesis is proposed that differences in population dynamics of the sea urchins may explain for this pattern. In the southern area mortality and individual growth rates may be higher than in the northern area because of highest temperature in the south. A higher growth rate may cause a shorter life length in the south than in the north. Then a higher and more regular recruitment is needed to stabilize a population in the south. If the population decreases to under critical breeding level, there is a greater chance for the sea urchin population to break down. A bet-hedging life history strategy is previously proposed for sea urchins. Bet-hedging, explained shortly, means that populations sometimes rapidly increase to a high level, but afterwards the density gradually decreases. This strategy is here discussed in the light of different stages of the life table and regional differences in the water temperature. Each parts of the life cycle should be analysed to find if the mortality at any of the stages is sensitive to fluctuations. High longevity of a species may compensate for high variability in recruitment.

**A REVIEW OF QPX DISEASE EMPHASIZING INITIATION AND PROGRESSION.** Roxanna Smolowitz, Marine Biological Laboratory, 7 MBL St., Woods Hole, MA 02536.

QPX was reported by Drinnan and Henderson in hard clams from Canada in 1960. In 1995, QPX was diagnosed in cultured hard clams in Provincetown and Duxbury, MA. QPX has since been identified in clams from Virginia, New Jersey, New York and Rhode Island waters, as well as other locations in Massachusetts. It continues to cause marked mortality in MA and RI. QPX organisms primarily invade through the mantle at the base of the siphon. Studies have demonstrated direct infection of naive clams three months after exposure to infected dying clams, but not when exposed to cultured QPX via the water column. Infection location

in 100% of those clams occurred at the siphon base and in the adjacent mantle. In other work, seed clams examined six months after planting showed disease in 33% of the population and the primary infection site, identified histologically, was at the base of the siphon. QPX organisms were noted amid debris in the mantle cavity at the base of the retracted siphon (pseudofeces location). These findings indicate residence time in that location, perhaps as part of pseudofeces, is important in the pathogenesis of the disease. Quizzically, prevalence of QPX disease appears to vary greatly with some plots showing high prevalence in one clam population followed by low prevalence in a replacement population, or some plots repeatedly showing high prevalence of the disease even in replacement populations. Studies show disease prevalence at any single time period may not accurately indicate previous or future disease prevalence.

**GENETIC IDENTIFICATION AND PHYLOGENY OF THE UNIONID GENERA, *LAMPSILIS* AND *ELLIPTIO* OF THE SOUTH EAST ATLANTIC SLOPE.** Kristine M. Sommer, Ami E. Wilbur and Michael A. McCartney. University of North Carolina, Department of Biology and Marine Science, 601 S. College Road, Wilmington, NC 28403.

Approximately 70% of freshwater mussel species in North America are considered extinct, endangered, or threatened, and a large number of these are endemic to a narrow geographic range. Freshwater mussel conservation efforts have been limited by taxonomic ambiguities and morphologic convergence. Lake Waccamaw in southeastern North Carolina contains two described endemic species, *Lampsilis fullerkati* and *Elliptio waccamawensis*, which conchologically are nearly identical, complicating conservation efforts. To help alleviate this problem, a Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) assay was developed for genetic identification. Genomic DNA was obtained using a non-lethal method of hemolymph extraction. Two diagnostic RFLPs were used to type 112 individuals. RFLP and sequencing data showed that three individuals were misidentified based on morphology. In addition, phylogenetic analysis was used to assess the taxonomic status of these putative endemics. 16S ribosomal DNA sequences were obtained from 140 individuals from both genera, sampled from Lake Waccamaw, the adjacent Waccamaw River, and the Yadkin/Pee Dee and Lumber Rivers in the Pee Dee Drainage. Results from neighbor-joining and maximum likelihood trees suggest the endemic status of both *L. fullerkati* and *E. waccamawensis* may need to be reconsidered. *Lampsilis fullerkati* individuals fall in a large clade containing *Lampsilis radiata radiata* from outside the lake, and the endemic is not a distinct phylogroup. Additional, faster-evolving gene regions and type specimens are being sequenced to evaluate this result. A similar approach is being used for *E. waccamawensis*, which appears phylogenetically indistinguishable from *Elliptio* collected outside of the Lake.

#### INFLUENCE OF CLIMATIC CYCLES ON THE INFECTION OF EASTERN OYSTERS BY *PERKINSUS MARINUS*.

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*Perkinsus* (= *Dermocystidium*) *marinus* is a major cause of mortalities in eastern oysters, *Crassostrea virginica*. Since initiation of infection and progression of disease are favored by high temperature and high salinity, we hypothesized that climatic cycles influence cycles of disease. Using wavelet analysis and a 10-year data set of disease prevalence and intensity, and water temperature and salinity from a Louisiana site, we show a teleconnection between the El Niño Southern Oscillation and oyster disease in the northern Gulf of Mexico. Inter-annual variation is important in the initiation and intensification of disease, and salinity is the primary driving factor. An increase in salinity is followed in several months by an increase in prevalence followed in several months by an increase in infection intensity, with salinity shifts strongly driven by ENSO events. The time series suggests that epizootics can be initiated within six months of a La Niña event, during which high water temperature and high salinity coincide. This relationship makes it possible to anticipate epizootics of *P. marinus* from climate models and to better manage oyster populations.

#### DERMOWATCH DETECTS DISEASE HOTSPOTS IN THE GULF OF MEXICO.

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DermoWatch ([www.dermowatch.org](http://www.dermowatch.org)) is a web-based community for the monitoring and management of the lethal oyster parasite, *Perkinsus marinus* (= *Dermocystidium marinum*). Oysters from the major oyster producing areas of the Gulf of Mexico are collected and assayed for *P. marinus*. The web site displays station information, water temperature and salinity, and percent infection and disease intensity (weighted incidence) of sub-market and market-sized oysters. Weighted incidence values of 2.0 and greater are highlighted as "hotspots", where oysters (*Crassostrea virginica*) are likely experiencing serious disease-related mortality. Recent disease hotspots are found in Corpus Christi Bay, Aransas Bay, Matagorda Bay and Galveston Bay. Within a bay, hotspots are established on high-salinity reefs and proceed up-estuary. Between bays, hotspots tend to progress from south to north. Within and between bay patterns of progression and regression of disease hotspots are likely cyclical, and should provide a useful indicator of the trajectory of infection.

**INFLUENCE OF OYSTER REEF VERTICAL COMPLEXITY IN STRUCTURING SPECIES SPECIFIC INTERACTIONS AND TROPHIC LINKAGES.** Joseph Michael Sou-nier, M. H. Posey and T. D. Alphin. UNC-Wilmington, Center for Marine Science, 5600 Marvin K. Moss Lane, Wilmington, NC 28409.

Habitat complexity is important in regulating trophic linkages, with broad implications for community composition. Habitat complexity can alter species interactions through impacts on refuge and food. High and low complexity oyster reefs in southeastern North Carolina provide an ideal system to test these ideas. Interactions between bivalve prey (oysters and mussels) and predators (mud crabs) may be affected by the presence of vertical complexity and consequent access by larger predatory crabs. We examined the effects of oyster reef vertical complexity on mud crab populations and mud crab: bivalve interactions in the presence of a top predator, the blue crab. Mud crab density was significantly greater in areas of high vertical complexity during both field and lab studies. Lab studies indicated mud crab predation on bivalve (oyster spat and mussels) prey was greater in high complexity oyster patches, but consumption of mussels was greater than consumption of spat for all trials. In trials containing the top predator, predation by mud crabs was reduced, especially in the low complexity patches where predation approached zero. Field studies show oyster spat recruitment to be higher on newly created low complexity reefs. Tethering trials indicate predation on mud crabs increased as oyster reef complexity decreased with open sand having the highest predation. However, an edge effect was detected for high complexity reefs, with highest predation on mud crabs located within five cm of the edge. Reef complexity in this system may have limited top predator access, affecting mud crab: bivalve predator-prey dynamics.

**THE FEASIBILITY OF USING TRIPLOID *CRASSOSTREA VIRGINICA* FOR ON BOTTOM CULTURE IN THE CHESAPEAKE BAY.** Melissa Southworth<sup>1</sup>, Roger Mann<sup>1</sup>, Thomas Leggett Jr<sup>2</sup> and A. Erskine<sup>3</sup>. <sup>1</sup>Virginia Institute of Marine Science, P.O. Box 1346, Gloucester Point, VA 23062, <sup>2</sup>Chesapeake Bay Foundation, <sup>3</sup>Cowart Seafood Company and Bevan's Oyster Company.

The Federal Register, Vol. 69, No. 2, dated Monday, January 5, 2004 provided notice of "DEPARTMENT OF DEFENSE, Department of the Army; Corps of Engineers Intent [To Prepare a Programmatic Environmental Impact Statement] for the Proposed Introduction of the Oyster Species, *Crassostrea ariakensis*, Into the Tidal Waters of Maryland and Virginia To Establish a Naturalized, Reproducing, and Self-Sustaining Population of This Oyster Species," and described seven alternatives to be evaluated in the EIS process. Alternative 4 addressed the issue of using the native oyster (*Crassostrea virginica*) species in the development and expansion of aquaculture in the Chesapeake Bay. In the past, the ma-

jority of Virginia's commercial oyster production came from leased oyster ground. Historically, lease holders would either purchase or harvest oyster seed from rivers such as the Great Wicomico, Piankatank or James, and plant on their leases throughout the Virginia portion of the Bay. We describe a project to examine the feasibility of using triploid native spat on shell in the same manner as oyster planters have used wild oyster seed from seed Rivers. Approximately forty million triploid larvae were set on 400 bushels of shell and planted on an oyster bed owned by Bevan's Oyster Company in the Yeocomico River, Virginia in summer 2005. We present here early results of setting success, growth, mortality and condition index.

**COMMUNITY ECOLOGY OF FRESHWATER MUSSEL BEDS: THE ROLE OF TEMPERATURE AND DOMINANT SPECIES ON COMMUNITY STRUCTURE AND RENDERED ECOLOGICAL SERVICES.** Daniel E. Spooner and Caryn C. Vaughn, University of Oklahoma.

Recent work suggests that freshwater mussel beds significantly influence stream ecosystem function through benthic-pelagic coupling of energy and nutrients. Mussel communities comprise a mosaic of species that differ in their species-specific thermal performance. These performance curves influence resource acquisition, potentially shaping community structure (species dominance) and subsequent ecological function. We traveled to 21 mussel beds across southeastern Oklahoma and western Arkansas. We quantified community structure (time-search and quadrats), physiological condition (glycogen, body condition index (BCI), and measures of rendered services (metabolism, nutrient excretion). We predicted that species would be dominant at sites that closely resemble their empirically derived thermal performance optima. Our results to date suggest that communities are alternately dominated by two species *Actinonaias ligamentina* and *Amblema plicata*. Both species co-occur, have opposing thermal performance trajectories, and are negatively correlated within sites. In addition, condition and performance indices of co-occurring mussel species was related to species richness, *A. ligamentina*, and *A. plicata* biomass. These results suggest that species interactions including facilitation/competition may be important factors explaining community structure and subsequent ecological services rendered by freshwater mussel beds.

**OVIERY IN THE SPOT SHRIMP (*PANDALUS PLATYCEROS*) OF HOOD CANAL.** David A. Sterritt, Washington Department of Fish and Wildlife.

Egg numbers and seasonal trends in ovigery of were established for Spot shrimp (*Pandalus platyceros*) from Hood Canal during testfisheries conducted during the 2003–2005 season. Ovigerous shrimp were found to be smaller in size and had fewer



eggs than Spot shrimp sampled in other investigations. A linear relation was found between egg number and carapace length.

**EMBRYONIC DEVELOPMENT AND MORPHOMETRY OF BLUE KING CRAB *PARALITHODES PLATYPUS*, STUDIED BY IMAGE ANALYSIS.** Bradley G. Stevens, NMFS/NOAA Kodiak Fisheries Research Center.

Embryonic development has been described for few commercial crab species, and no standard exists for defining developmental stages. I examined the embryonic development of the blue king crab, *Paralithodes platypus*, from the Pribilof Islands in the eastern Bering Sea. Fertilized embryos were digitally photographed at various intervals throughout their development using a compound microscope and measured with an image analysis program. Seven morphometric parameters were measured (total area, yolk area, embryo length and width, average diameter, eye length and width) and four indices were calculated (percent yolk, ellipticity, elongation, and circularity). First divisions occurred on day four, after which divisions occurred daily until the blastopore appeared at day 28. A "V"-shaped embryo became apparent on day 114, and the eyes became pigmented by day 192. Hatching occurred from day 381 to day 409, and required at least 33 days. Embryo area declined from 0.95 mm<sup>2</sup> on day 1 to 0.83 mm<sup>2</sup> on day 72, and then increased to 1.28 mm<sup>2</sup> on day 388. Developmental stages were defined visually and by using cluster analysis of embryo measurements. Both methods resulted in an optimum selection of 12 stages. Visual methods were better at defining early changes, but morphometric measurements were better at defining middle and later stages. Morphometric analysis techniques may lead to improved understanding of crustacean embryogenesis and effects of environmental change, and have applications in the aquaculture industry.

**PRELIMINARY ANALYSES OF GENETIC STRUCTURE WITHIN AND AMONG REMNANT POPULATIONS OF THE OLYMPIA OYSTER, *OSTREA CONCHAPHILA*.** David A. Stick<sup>1</sup>, Heather Hunsperger<sup>2</sup>, Chris Langdon<sup>1</sup>, Michael A. Banks<sup>1</sup> and Mark D. Camara<sup>3</sup>. <sup>1</sup>Oregon State University, COMES, HMSC, 2030 SE Marine Science Dr., Newport, OR 97365, <sup>2</sup>Eckerd College, St. Petersburg, FL, <sup>3</sup>USDA-ARS, HMSC, Newport, OR.

The Olympia oyster, *Ostrea conchaphila*, is the only oyster species native to the Pacific Northwest. Historically, the species ranged from Southeastern Alaska to Baja, California, Mexico and supported both Tribal subsistence fisheries and large commercial harvests. Over-exploitation, habitat degradation, and competition and predation from non-native species have drastically depleted densities and reduced the overall range, but remnant populations persist. Due to the species' historical significance and the ecological services provided by oyster reef habitats, numerous restoration

efforts are proceeding without a full understanding of existing population structure, which may be complicated due to *O. conchaphila*'s larviparous mode of reproduction and limited larval dispersal as well as extensive population admixture due to human mediated translocations. Identifying appropriate broodstock and maintaining the genetic integrity of populations is essential for the long-term success of restoration efforts, but conducting population-level studies have been hindered by a lack of molecular markers. We have developed a number of microsatellite DNA markers in *O. conchaphila* and conducted preliminary analyses of genetic structure. We first constructed several microsatellite enriched genomic libraries then isolated and sequenced individual clones from them. We next designed primers to the flanking regions of confirmed microsatellites, optimized PCR conditions and verified Mendelian segregation in larvae from five hatchery-produced full sib families. Polymorphism was assayed using a panel of 96 oysters from eight locations, and the most polymorphic markers were used for preliminary analyses of broad-scale patterns of genetic diversity and differentiation within and among populations ranging from northern Vancouver Island, BC to San Francisco Bay, CA.

**IMPACTS OF OYSTER REEF ARCHITECTURE ON SPECIES DIVERSITY AND PREDATION.** Jennifer L. Stiner and Linda J. Walters, University of Central Florida.

Widely regarded as a keystone species and ecosystem engineer, the eastern oyster, *Crassostrea virginica*, plays a vital role in estuarine environments. Complex three-dimensional oyster reefs act as biodiversity havens. Recently, concern for this resource has arisen in Mosquito Lagoon, Florida. Since the 1990s, intense boating activity has caused atypical dead margins (mounds of disarticulated shells) to emerge on the seaward edges of oyster reefs located along major boating channels. Once dead margins are formed, little is known about their influence on biotic composition and interactions. This study focused on the impact of dead margins on (1) species diversity and (2) predation. For one year, monthly surveys were conducted using lift nets to document all mobile species present on reefs in Mosquito Lagoon. Sixty-four species were recorded, including fishes, crustaceans, molluscs, echinoderms, and worms. Mini lift net trials revealed species diversity to be highest on fore-reef and back-reef areas, with drastic reductions on dead margins of impacted reefs. Field experiments were conducted to determine the impact of dead margins on the vulnerability of juvenile oysters. Structural variables (e.g. shell orientation, single versus clumped shells, and overall slope) were manipulated and effects were observed on oyster mortality and predator maneuverability. Responses of three major predators (*Callinectes sapidus*, *Urosalpinx cinerea*, and *Panopeus herbstii*) differed. For *C. sapidus* and *P. herbstii*, predation was higher on disarticulated shells while *U. cinerea* showed a preference for spat on clumped shells. Overall, this study documents ecological implications of dead margins on reefs of *C. virginica*.



**VARIATION IN OYSTER SHELL STRENGTH BASED ON STOCK ORIGIN, SHELL SIZE AND TRANSPLANT EFFECT.** Heather Stoker, Troy Alphin and Martin Posey. University of North Carolina, Center for Marine Science, 5600 Marvin K. Moss Ln., Wilmington, NC 28409.

This study compares the breaking strength of *Crassostrea virginica* shells from four Southeastern North Carolina estuarine systems: New River, Cape Fear River, Stump Sound and White Oak River. Shell strength comparisons were made among three size classes within each estuary and among estuaries for oysters in one size class (40–50 mm). Five oysters per size class per estuary (120 oysters total) were selected. As morphological differences may suggest variation in mechanical properties, the length, width, muscle scar thickness and weight of each shell were recorded. An eleven-millimeter disc was cut from the muscle scar of the right valve of each specimen. The discs were strength tested using an Instron 8511 material testing machine, measuring compressive break strength (MN/m<sup>2</sup>). The oysters used in this project were from a previous study on stock differentiation; therefore, shell strength can be compared to existing data on the oysters' growth, survival, stock type and estuarine conditions. If the intrinsic strength of *C. virginica* shells differs among origin or transplant location, this has implications of potential differences in predation risk and susceptibility to disease.

**POPULATION STRUCTURE AND PHYLOGEOGRAPHY OF THE PINTO ABALONE (*HALIOTIS KAMTSCHATKANA*) IN WASHINGTON STATE.** Kristi Straus, Kerry Naish and Carolyn Friedman. University of Washington, School of Aquatic & Fishery Sciences, P.O. Box 355020, Seattle, WA 98195.

The Pinto abalone (*Haliotis kamtschatkana*) is the predominant haliotid species in Washington State and is an ecologically important herbivore in rocky subtidal habitats. Although the Washington abalone fishery was closed in 1994, abundance and population densities have declined dramatically since this time. In order to enact an effective conservation strategy, it is imperative to determine the population structure of the Pinto abalone. Previous microsatellite studies indicated low differentiation among Pinto abalone collections in British Columbia, Canada. However, our research on genetic variation at microsatellite loci in over 100 individuals from Washington State revealed substantial population structure. All microsatellite loci exhibited significant deviations from Hardy Weinberg expectations (positive  $F_{IS}$ ) and linkage disequilibrium was evident between most pairs of loci. Using MtDNA sequence analysis, ten individuals from S.E. Alaska (U.S.), British Columbia (Canada), and Washington State (U.S.), and five individuals from California (U.S.) were sequenced at the Cytochrome Oxidase B locus (Cyt B, 404 bp). Results from this locus indicate that although animals from Alaska, B.C. and California appear to be closely related, six of the ten Washington individuals sequenced

diverge strongly. These six individuals share four haplotypes unique to Washington State. These same six individuals were sequenced at Cytochrome Oxidase I (COI, 343 bp). At COI, these six individuals share three unique haplotypes relative to sequence available on Genbank. Further studies are needed to elucidate the biological significance of the observed genetic differences. These findings have significant implications for the listing and restoration of abalone found in Washington State.

**GENETIC ISSUES IN FLORIDA HARD CLAM AQUACULTURE.** Leslie Sturmer<sup>1</sup>, Charles Adams<sup>2</sup>, Patrick Baker<sup>2</sup>, Shirley Baker<sup>2</sup>, Elise Hoover<sup>2</sup>, Claudia Rocha<sup>2</sup> and John Scarpa<sup>3</sup>. <sup>1</sup>Cooperative Extension Service, University of Florida, Cedar Key, FL 32625; <sup>2</sup>Institute of Food and Agricultural Sciences, University of Florida, Gainesville, FL 32611; <sup>3</sup>Harbor Branch Oceanographic Institution, Ft. Pierce, FL 34946.

The hatchery-based hard clam aquaculture industry in Florida, while rapidly growing, is in its infancy in terms of stock management and improvement. A concern in the loss of useful genetic variation through unintentional inbreeding was addressed by developing an index of clam stock health. Using mitochondrial DNA and microsatellite loci in the nuclear genome, genetic diversity in captive strains from multiple Florida hatchery stocks was quantified and compared to wild stocks. Hatchery stocks were cultured simultaneously during 2004–5 to compare performance under aquaculture conditions and correlate with genetic diversity. The need for a hardier clam strain also became evident as Florida culturists reported below average survivals during hot summers. The project team is currently evaluating triploidy as a means to improve stress resistance by reducing gametogenesis and increasing body mass. Triplicate groups of sibling diploid and triploid hard clams were planted under commercial conditions on leases to compare production characteristics. Responses of clams subjected to laboratory environmental challenges (high water temperatures and low dissolved oxygen levels) are being evaluated to determine the physiological mechanism by which triploidy may improve survival in subtropical waters. Finally, the economics of triploid seed production and triploid clam culture will be characterized.

**A SURVEY OF MARINE COMMUNITIES IN ALTERNATIVE CULTURE METHOD OYSTER AND CLAM SITES.** Andrew D. Suhrbier<sup>1</sup>, Daniel P. Cheney<sup>1</sup> and Jonathan P. Davis<sup>2</sup>. <sup>1</sup>Pacific Shellfish Institute, 120 State Ave. NE #142, Olympia, WA 98501. <sup>2</sup>Baywater Inc.

The past two decades have seen a rapid expansion of alternative and innovative methods for the cultivation of oysters and hardshell clams; however, there is limited information on the interactions of these practices with the surrounding environment. As part of a larger project, multi-season sampling was conducted to 1) assess and compare benthic infauna and epifauna species diversity and

density, across habitat types within culture and control sites; to 2) assess responses of infauna and epifauna to culture conditions; and to 3) monitor and compare selected macrofauna including juvenile salmon, shrimp and crabs, across habitat types. Methods evaluated included bag-on-rack, bag on bottom, and suspended bag culture of oysters and bag on bottom and net-protected ground culture of manila clams. Discussed will be the results of seasonal benthic collections, net deployments and underwater video footage at each survey site. Survey sites included commercial shellfish farms in Thorndyke Bay, Hood Canal and Eld Inlet, in South Puget Sound, Washington. This project was funded by the SeaGrant National Marine Aquaculture Initiative.

**CONNECTICUT'S HARD CLAM INDUSTRY AND QPX-DISEASE.** Inke Sunila, State of CT, Department of Agriculture, Bureau of Agriculture.

Connecticut, on Long Island Sound's north shore, is the leading producer of hard clams, *Mercenaria mercenaria*, with a yearly production of over 400,000 bushels and a yearly farm gate value of over \$10 million. Since 1997, the year of a major *Haplosporidium nelsoni* (MSX) epizootic among oysters, production has been rising steadily. After that harvesters concentrated on the hard clam fishery. Hard clam aquaculture is practiced on over 65,000 acres of leased grounds. Recruitment originates mostly from natural set and harvest size clams are collected by hydraulic dredges and transplanted onto approved grounds for depurification prior to marketing. The health of CT's clams has been monitored annually since 1997. QPX was detected in six out of 2358 clams (0.3%) from 77 different samples along the shoreline. None of the QPX-positive clams originated from commercial clam grounds. Two positive clams were collected from natural oyster beds during culching. Three positive clams were collected next to shipping channels. One of the clams originated from a bed of false quahogs, *Pitar morrhuana*, being surveyed as a potential future hard clam site. Sampling sites for the positive clams are distributed along the entire CT shoreline: Bridgeport, Stratford, New Haven and Groton. Histopathologically affected clams have mucus producing, proliferating presentation of QPX-disease. However, QPX is not currently considered to pose a threat to CT's hard clam industry.

**THE LOUISIANA OYSTER RECOVERY PLAN: A RESPONSE TO HURRICANES KATRINA AND RITA.** John Supan<sup>1</sup> and Mike Voisin<sup>2</sup>. <sup>1</sup>Louisiana Sea Grant College Program, LSU, Baton Rouge, LA 70803. <sup>2</sup>Louisiana Oyster Task Force, Motivaitt Seafood, Inc.

Hurricane Katrina caused unprecedented damage to the central gulf coast, being the greatest natural disaster in U.S. history. The paths of Hurricanes Katrina and Rita passed through major Louisiana oyster production areas, causing between 55–70% oyster mortality to the public grounds by scouring and overburden, with

likely similar damage to private farms yet to be determined. Infrastructure and fleet damage to public agencies and the private sector were also severe. The Louisiana Oyster Task Force, legislatively mandated to guide the state in the management and development of its oyster industry, is enacting a recovery plan to address five areas of need: (1) vessel and lock recovery; (2) harvest areas, (3) dock and unloading facilities, (4) processing plants, and (5) market development. This multi-faceted approach challenges all involved to new paradigms. Some of the components include: refloating some 1,800 commercial fishing vessels aground by storm surges; continued and additional microbiological sampling to open more harvest areas; developing dockside refrigeration program; cultch placement on public and private oyster grounds; opening and closing oyster seasons on public oyster grounds by location and specific dates; lifting current moratorium on new oyster farm lands; rebuilding and improve the Sea Grant oyster hatchery on Grand Isle and initiate a Louisiana Oyster Seed Program focusing on genetically superior stocks; developing off-bottom culture in designated marine enterprise zones and/or aquaculture parks; and, postponing 2005 lease rental payments until 2006.

**BIOCHEMICAL COMPOSITION AND ADDUCTOR MUSCLE CELL SIZE OF TRIPLOID AND DIPLOID BAY SCALLOP *ARGOPECTEN IRRADIANS*.** Amandine Surier<sup>1</sup>, Chester B. Zarnoch<sup>2</sup> and Richard C. Karney<sup>1</sup>. <sup>1</sup>Martha's Vineyard Shellfish Group, P.O. Box 1552, Oak Bluffs, MA 02557. <sup>2</sup>AREAC, Brooklyn College, Brooklyn NY 11210.

Triploidy, which has been found to improve overall performance in many shellfish, is particularly interesting in scallops as the increased growth rate is associated with decreased gonad development and increased adductor muscle mass. Triploid's increase in somatic biomass is thought to be the result of energy reallocation from reproduction towards somatic growth or increased fitness due to higher heterozygosity. Another hypothesis is triploidy gigantism, which associates the increased size of triploid organisms with a larger cell size. In July 2003, under funding from the Northeastern Regional Aquaculture Center, triploidy was induced in the bay scallop *Argopecten irradians*. The experimental scallops, which tested 97% triploid in July 2004, were grown in bottom cages in Katama Bay, Massachusetts. Triploid wet adductor muscle indexes were 52% and 17% greater than diploids in July and October 2003 respectively.

In December 2004, triploid and diploid adductor muscle samples were sent to Brooklyn College for biochemical composition. Although the protein and carbohydrate contents were higher and lipid content was lower in triploid muscles the differences were not significant. In the spring 2005, survivors from the experiment were sampled for cell size analysis. A portion of the smooth adductor muscle from triploids and diploids was processed using standard histological techniques, photographed and analyzed

for muscle cell size and number of cells/area using NIH ImageJ software. Adductor smooth muscle fibers of triploids were significantly wider than those of diploid controls. In contrast, the number of muscle fibers per unit area was not significantly different between ploidy groups.

**EFFECTS OF PREDATION AND SETTLEMENT SUBSTRATE CHOICE ON THE POST-SETTLEMENT SURVIVAL OF RED ABALONE, *HALIOTIS RUFESCENS*.** Clara Svedlund<sup>1</sup>, Steven Gaines<sup>1</sup>, Jennifer Caselle<sup>1</sup> and Laura Rogers-Bennett<sup>2</sup>. <sup>1</sup>Ecology, Evolution and Marine Biology, U. C. Santa Barbara, <sup>2</sup>California Department of Fish and Game & Bodega Marine Lab.

Historically abundant populations of abalone (*Haliotis* spp.) are now at drastically reduced levels throughout Southern California. Two particularly vulnerable stages in the life cycle of these broadcast spawning invertebrates are the larval and post-settlement stages. Laboratory studies of predation on cultured veliger larvae and newly settled red abalone (*Haliotis rufescens*) indicate that predation may be an extremely important source of mortality at these stages. Results from predation experiments revealed that important predators of *H. rufescens* include the crabs *Pagurus* sp., *Cancer* sp., and *Scyra* sp., the terebellid *Eupolytnia* sp., and the sea urchin *Strongylocentrotus purpuratus*. Ninety-seven percent of all predators tested were sources of mortality for settling or settled abalone, whether due to consumption, bulldozing, or other modes. Settlement experiments using three categories of encrusting red algae (ERA) indicated that substrate type may play a larger role in the survival and subsequent growth of settled larvae. Compared to the two other types, settlement and post-settlement survival was significantly higher for the category of crustose coralline algae (CCA) with an irregular surface (e.g. *Lithothamnium pacificum*). Further experiments that tested the interaction of substrate (the three ERA types) and predation (using two different predators) revealed that the more rugose substrate again conferred the highest survival. This higher survival may be due to the morphology of the CCA, which might provide abalone with predation refugia and/or superior grazing sources. Potential larval/post-larval seeding experiments may benefit from selection of sites with a higher cover of the rugose encrusting corallines, and exclusion of probable predators.

**ANNUAL VS. NONANNUAL EGG EXTRUSION, MATING VS. UTILIZATION OF STORED SPERM, AND LARVAL HATCHING OF KODIAK, ALASKA DUNGENESS CRAB.** Katherine M. Swiney, NOAA Fisheries, Alaska Fisheries Science Center.

Previous studies report nonannual egg extrusion among Dungeness crab in southeastern Alaska, which differs from the annual egg extrusion typically reported for Dungeness crab. Dungeness

crab from Kodiak, Alaska waters were reared in flow-through tanks to determine if egg extrusion is annual and to observe which crab mate or utilize stored sperm to fertilize egg clutches. Females were collected from two bays on the eastside of Kodiak Island May through August 2005, a time in which females should either have eggs or recently hatched larvae. Thirty one percent of females collected did not have eggs the previous reproductive season. Seventy one percent of females brought into the laboratory used stored sperm to fertilize eggs while 26% molted/mated. Females that molted/mated were significantly smaller than females that did not molt. As of 12, December 2005, 52% of females extruded eggs in two consecutive years, whereas 28% skipped at least one reproductive season and extruded an egg clutch the following season; more extrusion is expected. Larval hatching was examined in 2004 with females brought into the laboratory a week before hatching, which began at the end of May and lasted 25 days. Individually, larval hatching ranged from 8 to 16 days with a mean of 11 days. Larger crab took significantly longer to hatch larvae and had significantly more larvae. Egg extrusion is not annual for all mature Dungeness crab collected from Kodiak waters, however, preliminary evidence suggests more crab may extrude eggs annually in the Kodiak area than in southeastern Alaska.

**CAROTENOIDS IN THE SEA URCHIN *PARACENTROTUS LIVIDUS*.** Rachael Symonds and Andrew Young, Liverpool John Moores University, School of Biological and Earth Sciences, Byron St., Liverpool UK.

The roe of the edible sea urchin *Paracentrotus lividus* is commercially valuable and highly prized in the sushi market. However, roe colour varies with season, the urchin's nutritional state, its state of maturity and its sex. This study has characterised the carotenoid pigment profile of the gonads and gut wall of wild *P. lividus* using a combination of reversed-phase diode-array HPLC and LC-MS. The major pigments occurring in the gut wall are breakdown products of fucoxanthin, namely fucoxanthinol and amarouciaxanthin A. Lower levels of echinenone, lutein, isozeaxanthin and  $\beta$ -carotene are also present. The gut wall of the female urchins contains pigments not present in the males, indicating additional metabolism in the former. It is clear that the gut wall is a major site of carotenoid metabolism. Male and female gonads have a broadly similar pigment profile with echinenone accounting for between 70–80% of the total carotenoid (c.f. gut: 20%). Both all-*trans* and 9'-*cis* forms of  $\beta$ -echinenone are found in the ratio 1:10 (w/w). The detection of large levels of 9'-*cis*-echinenone in wild *P. lividus* is unexpected due to the absence of 9'-*cis* forms of carotenoid in the natural, algal, diet. Amounts of lutein and isozeaxanthin are consistently higher in the female rather than male gonads with levels of these peaking shortly after spawning. The presence of 9'-*cis* echinenone as the major carotenoid contributing to the pigmentation/colour of the gonad is an important observation in terms

of developing artificial diets for urchin cultivation. The implications of this are discussed further.

**EELGRASS RESPONDS TO OYSTERS AND GROW-OUT METHODS IN AN AQUACULTURE SETTING.** Heather Tallis<sup>1</sup>, Jennifer Ruesink<sup>1</sup>, Brett Dumbauld<sup>2</sup>, Sally Hacker<sup>3</sup> and Lorena Wischart<sup>3</sup>. <sup>1</sup>University of Washington, <sup>2</sup>Hatfield Marine Science Center, <sup>3</sup>Oregon State University.

Aquaculture has been shown to have negative impacts on eelgrass and certain aquaculture practices have been banned in the Pacific Northwest (USA) to protect eelgrass as valuable habitat. We argue that the magnitude of tradeoffs between aquaculture and biodiversity depends on the ecological details of the production system. We explored three aquaculture systems (dredged on-bottom, hand picked on-bottom, long lines) to quantify the tradeoffs between oyster (*Crassostrea gigas*) aquaculture and eelgrass (*Zostera marina*) habitat. Capitalizing on large scale “manipulations” for aquaculture in Willapa Bay, WA, we found lower eelgrass density in all aquaculture systems relative to uncultivated areas in 2002–2004. Dredged beds had the lowest eelgrass densities (~50% < uncultivated) while hand picked and long line beds were intermediate. After experimental dredging, eelgrass densities recovered in one to four years, depending on site and disturbance intensity. Additionally, eelgrass density declined with oyster density in cultivated beds, although oysters were sparsely planted (~20% cover). Eelgrass growth ( $\text{g g}^{-1} \text{d}^{-1}$ ), measured in 2004, also varied by aquaculture type and oyster density, although patterns in this individual-level indicator were context-specific. Total above-ground production was largely driven by variation in eelgrass density, so dredged beds had the lowest production. Our findings do not support regulations against all on-bottom aquaculture because hand picked beds showed the least tradeoff in terms of eelgrass production and were 1.5–2.3 times more productive than dredged beds. We suggest several management options that may allow the continued co-existence of aquaculture and eelgrass in the Pacific Northwest.

**SETTLEMENT OF *CRASSOSTREA ARIAKENSIS* LARVAE UNDER CONDITIONS FOUND IN THE CHESAPEAKE BAY.** Mario N. Tamburr<sup>1</sup>, Mark W. Luckenbach<sup>2</sup>, Denise L. Breithurg<sup>3</sup> and Stephanie M. Bonniwell<sup>4</sup>. <sup>1</sup>Chesapeake Biological Laboratory/UMCES, <sup>2</sup>Virginia Institute of Marine Science, <sup>3</sup>Smithsonian Environmental Research Center, <sup>4</sup>Virginia Institute of Marine Science.

The Asian oyster (*Crassostrea ariakensis*) is being considered for introduction into the Chesapeake Bay. However, our current understanding of the biology and ecology of *C. ariakensis* is insufficient to predict whether an introduction will be successful,

provide desired benefits, or have adverse impacts. Behavior of native oyster (*C. virginica*) pediveligers has been studied for many years and it is well established that they use a variety of habitat characteristics when selecting a site for colonization. Perhaps the most important of these are chemical cues emitted by adult conspecifics, which can lead to gregarious larval settlement and dense, persistent reef communities. Conversely, almost nothing is known about how larvae of *C. ariakensis* respond to conditions found in Chesapeake Bay or about the critical life history processes of settlement and metamorphosis. We have examined how the behavior and substrate preference of two *C. ariakensis* strains (“south China” and “west coast”) at the time of settlement compares with that of *C. virginica*. Results demonstrate many similarities but also a few important differences. For example, both species and strains of larvae greatly prefer natural substrates (e.g. shell) covered with biofilms for colonization but the west coast strain of *C. ariakensis* exhibited greater attachment onto manmade substrates (e.g. fiberglass) than *C. virginica*. Waterborne chemical cues emitted by adult oysters were also found to enhance substrate attachment for all larval forms, whereas initial data suggests hypoxia inhibits larval attachment with the south China strain of *C. ariakensis* perhaps most sensitive to low oxygen conditions.

**ASSESSMENT OF RED ABALONE, *HALIOTIS RUFESCENS*, POPULATIONS AT SAN MIGUEL ISLAND, CALIFORNIA.** Ian K. Taniguchi and Peter L. Haaker, California Department of Fish & Game.

San Miguel Island contains the southernmost large population of red abalone, *Haliotis rufescens*, along the Pacific coast, but commercial and recreational abalone fishing was closed in 1997 because of a general decline. Since then, red abalone size frequency has improved at San Miguel Island and at some surrounding locations. The California Fish and Game Commission have recently adopted an Abalone Recovery and Management Plan, which includes a provision for reopening a commercial and recreational fishery at San Miguel Island. To address the potential fishery, an assessment of the abalone resources will need to be completed. Diving surveys, using  $2 \times 30$  m band transects to collect density and size frequency data, are optimized using stratification of suitable abalone habitat at San Miguel Island within one nautical mile square ( $1 \text{ min} \times 1 \text{ min}$ ) grids. Maps of giant kelp, *Macrocystis pyrifera*, beds are used as a surrogate for hard benthic abalone habitat, and are overlain on the one mile square grids in a geographic information system. Within the common grid-kelp areas, thirty points are randomly selected for diving surveys. Data will be used to determine red abalone size frequency and density for management, and compared to historic size frequency data. It will also provide comparisons between areas in and out side of recently established marine protected areas.

**EVIDENCE OF A RESPONSE TO UNINTENTIONAL SELECTION FOR FASTER DEVELOPMENT ASSOCIATED WITH INBREEDING DEPRESSION IN *C. GIGAS*.** Nicolas Taris<sup>1</sup>, Frederico M. Batista<sup>2</sup>, Eric Marissal<sup>1</sup> and Pierre Bondry<sup>1</sup>. <sup>1</sup>INFREMER-LGP, Station de la Tremblade, La Tremblade, France. <sup>2</sup>INIAP/IPIMAR, Olhão, Portugal. <sup>3</sup>Grainocéan, St Martin de Ré, France.

Direct and indirect consequences of selective breeding in marine bivalves still remain largely unexplored. For species with two-phase life cycles, like the Pacific oyster (*Crassostrea gigas*), most studies have focused on juvenile and adult stages, but relatively few have considered the larval stage, especially in a domestication context. We assessed the impact of hatchery practices on larval traits, notably on larval growth (due to the culling of slow growing larvae), by the study of larval progenies. Larvae originating from crosses using parental oysters both from natural beds and from hatchery broodstock which had been selected for adult growth and shell shape for seven generations. A set of three microsatellite loci was used to compare genetic variability in the two parental broodstocks and to establish the relatedness between pairs of individuals within each broodstock. The mean relatedness was nearly six times higher in the hatchery broodstock than in the wild broodstock. On one hand, our results show a lower mean survival associated with larger variation of growth rate showing a bimodal distribution for the hatchery larval population. On the other hand, a higher success at metamorphosis was observed for the surviving larvae bred from hatchery parents. The results suggest that some of these larvae exhibited inbreeding depression but this was balanced by an overall positive response to selective pressures for larval growth.

**EVALUATION OF SUSPENDED ADPI® BAGS VS. BOTTOM PLANTING FOR USE AS BAY SCALLOP SPawner SANCTUARIES.** Stephen T. Tettelbach, Andrew Weinstock, Dennis Bonal, Chelsea Fitzsimons-Diaz, Richard Ames and Katherine Newman. Southampton College of Long Island University, 720 Northern Blvd., Brookville, NY 11548.

An off-bottom culture system for bay scallops (*Argopecten irradians irradians*), employing stacks of 15 mm mesh ADPI® bags (= arrays), was compared to bottom (free) planting as an alternate method for establishing spawner sanctuaries. Two stocks of hatchery-reared scallops were separately overwintered and then deployed at one site in Northwest Harbor, East Hampton, NY in ADPI arrays (50, 100, or 200 scallops/bag = 117, 234, or 468 scallops/m<sup>2</sup>, respectively) or free-planted at a mean density of ~95 scallops/m<sup>2</sup> on 30 March to 1 April 2005. In all, 12,600 scallops were stocked into ADPI arrays and ~235,000 scallops were free-planted. Overall survival, to late September 2005, of free-planted scallops (~31%) was better than that of scallops in ADPI bags (~18%). Cumulative mortality increased gradually in both scallop groups over the course of the study. Shell growth, from an initial mean stocking size of 38–40 mm, was significantly greater in free-planted scallops compared to those held in arrays ( $t = 7.10$ ;

$p < .0001$ ). The timing of spawning, as determined via biweekly monitoring of gonad indexes, was similar in both groups of scallops. After 4.5 months, biomass of epifaunal fouling organisms on free-planted scallops (mean = 0.88 g whole wet weight per scallop) was lower than that on scallops held in ADPI bags (mean = 3.78 g whole wet weight per scallop). A second field season in 2006 will be used to evaluate a modified version of the initial ADPI array system.

**VARIABILITY OF PHYTOPLANKTON BIOMASS IN THE DAMARISCOTTA RIVER ESTUARY.** Brian Thompson, Mary Jane Perry<sup>1</sup> and Christopher Davis<sup>2</sup>. <sup>1</sup>University of Maine, Darling Marine Center, 193 Clark's Cove Road, Walpole, ME 04573. <sup>2</sup>Pemaquid Oyster Company, P.O. Box 302, 1957 Friendship Road, Waldoboro, ME 04572.

Phytoplankton biomass and environmental parameters in the Damariscotta River Estuary, Maine, USA, were quantified over space and time to better assess the carrying capacity of this prime location for shellfish mariculture. Water samples were collected two to five times a week between February and October 2005 at three stations in the estuary—mouth, middle, and head—and analyzed for chlorophyll *a* and phaeopigment concentrations, dissolved inorganic nutrients, temperature, and salinity, with less frequent collection for phytoplankton taxonomic identification. In summer, additional locations were sampled from a small boat and with moorings. Stations between the middle and head of the estuary were occupied over two separate fortnightly tidal cycles between July and September; moored instruments were deployed from late August to early September. Photosynthetically available radiation was continuously recorded at the middle station at the Darling Marine Center dock. An offshore mooring, GoMOOS Buoy E, provided additional data to assess external forcing on the estuary. In 2005, the late winter phytoplankton bloom at the middle station was delayed and significantly smaller in both magnitude and duration in comparison to blooms in the previous two winters. Nitrate concentrations were highest at the beginning and end of the year, with a significant draw down of all nutrients concurrent with the late winter bloom. Chlorophyll concentrations were typically highest at locations in the upper estuary. Identification of regions with persistent occurrence of high phytoplankton biomass throughout the growing season are important to oyster farmers for determining locations for culturing.

**GENETIC AND ECOLOGICAL INTERACTIONS IN A MUSSEL HYBRID ZONE.** R. J. Thompson, D. J. Innes, J. B. Lowen and M. B. Miranda. Ocean Sciences Centre, Memorial University of Newfoundland.

Understanding the processes that maintain the coexistence of closely related species requires studies of life-history variation, competition and hybridization. *Mytilus edulis* and *M. trossulus* coexist and hybridize throughout Atlantic Canada. Nuclear and mtDNA genetic markers have shown that  $F_1$  hybrids are very rare

(<1% of the population) but individuals of mixed genotype (backcrosses) account for about 25%. Larvae and small mussels are predominantly *M. trossulus* or hybrids whereas larger mussels are almost exclusively *M. edulis*. A cohort analysis of laboratory-produced mussels of known species composition transplanted to the field showed that *M. edulis* grew faster and had a greater survival rate than *M. trossulus* or hybrids. A detailed analysis of energy allocation to soft tissues and shell demonstrated that *M. trossulus* had a greater investment in reproductive tissue and a lower investment in shell mass than *M. edulis*. Hybrids had an intermediate allocation pattern. A lower investment in shell mass, adductor muscle mass and byssus production resulted in a greater susceptibility of *M. trossulus* to crab and starfish predation. Life-history trade-offs involving reproduction, growth and survival are probably instrumental in explaining the coexistence of *M. edulis* and *M. trossulus*. Furthermore, a combination of reduced interspecific fertilization success (prezygotic) and post-fertilization mortality (postzygotic) results in partial reproductive isolation, which can explain the observed frequency of hybrids.

**MULTI-VARIATE ANALYSIS OF SPATIAL VARIABILITY OF OYSTER-REEF COMMUNITIES: THE INFLUENCE OF SALINITY.** S. Gregory Tolley, Aswani K. Volety, Mike Savarese, Christi M. Linardich, Laura D. Walls and Edwin M. Everham III. Florida Gulf Coast University, Coastal Watershed Institute, 10501 FGCU Blvd. South, Ft. Myers, FL 33965.

When assessing oyster-reef habitat in estuaries, it is important to understand the contribution of salinity to the spatial variability of associated organisms. How comparable is community structure among stations located along the salinity gradient of an estuary or among tidal tributaries experiencing different levels of freshwater inflow? To address these questions, multi-variate techniques were employed to analyze decapod crustacean and fish abundance data. Organisms were collected at three reefs along the salinity gradient (upper, middle, lower) of three estuaries: Caloosahatchee River, Estero River and Bay, and Faka Union Canal and Bay. Additional collections were made in Estero Bay from oyster reefs located at the mouths of five tidal tributaries. Cluster analysis did not reveal any natural groupings by estuary. Analysis of similarity did reveal significant differences among sites along the salinity gradients within estuaries and between Estero Bay tributaries. Multi-dimensional scaling identified community structure present at upper stations as distinct from that downstream, and at high-flow tributaries as distinct from that near low-flow tributaries. Upper stations and stations near high-flow tributaries were typified by the mud crab, *Eurypanopeus depressus*, and gobiid fishes. Downstream stations and stations near low-flow tributaries were typified by *E. depressus* and the porcelain crab, *Petrolisthes armatus*. Percent dissimilarity was greatest when upper and lower stations were compared or when high-flow and low-flow stations were compared. Variability in multivariate structure tended to be higher

upstream or in association with high-flow tidal tributaries, a pattern suggesting that freshwater inflow is disturbing communities at these locations.

**CAN HEAT SHOCK PROTEIN 70 EXPRESSION BE USED AS A BIOMARKER FOR ENVIRONMENTAL STRESS IN THE EASTERN OYSTER?** Nobuo Ueda and A. A. Boettcher. University of South Alabama, LSCB 124, Mobile, AL 36688.

Significant declines in eastern oyster, *Crassostrea virginica*, populations have occurred along the coasts of the eastern United States and Gulf of Mexico. Anthropogenic disturbances are thought to be the main cause of these declines, with changes in abiotic environmental conditions, either in conjunction with, or independent of these anthropogenic factors contributing to increased mortality in specific areas. Understanding the responses of oysters to environmental stressors and developing simple tools for detecting their effects is critical for the successful monitoring of oyster populations. As a part of the Alabama Oyster Reef Restoration Program, we have been examining the usefulness of heat shock protein 70 (HSP 70) expression as a biomarker of environmental stress in *C. virginica*. As has been shown with other oyster species, three isoforms of HSP 70, two constitutive (77 and 72 kDa) and one inducible (69 kDa), have been identified in the eastern oyster, *C. virginica*. However, it is not known how different environmental stresses impact the expression of these three isoforms. This study examined differences in expression of HSP70 isoforms in *C. virginica* based on both type of environmental stress (salinity, temperature, and dissolved oxygen) and age of the oyster. Levels of environmental stress, type of stress, and age of the oyster all impact HSP 70 expression, with differences in the expression levels among isoforms of HSP 70 associated with the different factors. Clarifying how these factors interact will allow for a better evaluation of the effectiveness of HSP 70 as a biomarker.

**PROTANDRY, MATURATION, AND SPAWNING IN CULTURED INTERTIDAL GEODUCK (*PANOPEA ABRUPTA*) CLAMS.** Brent Vadopalas<sup>1</sup>, Cyrus S. Y. Ma<sup>1</sup>, Jonathan P. Davis<sup>2</sup> and Carolyn S. Friedman<sup>1</sup>. University of Washington School of Aquatic and Fishery Science, 1122 NE Boat St., Seattle, WA 98105. <sup>2</sup>Baywater, Inc.

Among the challenges facing aquaculture of endemic species are potential negative effects of gene flow from cultured to wild populations. Solutions include maturation control, monosex outplants, spatial or temporal separation, and harvest before sexual maturation. To assess whether cultured intertidal geoduck clams mature and spawn before harvest, we sampled 12 geoduck ten times over the course of one year (2004–2005) from each of five year classes (1999–2003) planted conterminously in south Puget Sound, Washington. Additional sampling occurred through a second spawning season from the 2001, 2002, and 2003 year classes. Individuals were sexed, weighed, and measured; histological sections of gonad were classified by developmental stage (early ac-

tive, late active, ripe, partially spawned, spent). Using image analysis, we calculated a gonadal-somatic index for each individual. Of geoducks age 1.5–2.2 years, approximately 23% exhibited evidence of spawning and 71% were mature, compared to 68% and 89% respectively in geoducks age 4.5–5.0 years. We estimate that 50% maturation occurs at 64 mm shell length at this site. Male:female ratios in two to five year old geoducks were male-biased relative to the predominate 1:1 sex ratios observed in wild populations ( $p < 0.05$ ), providing evidence of facultative protandric dioecy. Maturation stage in cultured geoducks was temporally correlated with maturation stage in wild populations, indicating no temporal separation. Taken together, these data suggest that (1) pre-maturation harvest to prevent gene flow may be untenable and (2) future efforts be directed toward exploring the viability of maturation control in this species.

**USING QUANTITATIVE PCR AND DNA BARCODES TO QUANTIFY AND IDENTIFY MARINE INVERTEBRATE LARVAE.** Brent Vadopalas, Joshua V. Bouma, Chemine R. Jackels and Carolyn S. Friedman. University of Washington School of Aquatic and Fishery Science, 1122 NE Boat St., Seattle, WA 98115.

Information on the relationship between behavior and dispersion in marine invertebrate larvae has been hampered by the lack of suitably rapid techniques for identification and quantification from field samples. The need to understand larval dispersal dynamics of the pinto abalone (*Haliotis kamtschatkana*), which was recently listed as a species of concern (U.S.) and threatened (Canada), motivated the development of a high-throughput method for identification and quantification of abalone larvae in seawater samples. Seawater was filtered through a 70  $\mu$ m screen and DNA was extracted from all organisms present in sample retentate. Species-specific Cytochrome Oxidase I (COI) mitochondrial DNA sequence was used as template for Quantitative PCRs (QPCR) containing primers and a dual-labeled hydrolysis probe. Direct enumeration of larvae using light microscopy was used to verify quantity estimates derived from QPCR standard curves. To avoid bias from either underestimation of quantity or false negatives, abalone-specific reactions were multiplexed with primers, probe and exogenous template as an internal positive control (IPC) to indicate partial or full PCR inhibition. Differences between direct counts ( $n = 1-141$ ) and QPCR estimates were not significant, and the average coefficient of variation was 0.56 for within-run replicates, indicative of high accuracy and precision. We estimate that 80 samples can be processed from DNA extraction through QPCR in about four hours at a cost of approximately \$2/sample. Our results demonstrate that QPCR may have high utility for high throughput identification and quantification of specific marine invertebrate larvae in seawater samples via counts of mtDNA COI "barcodes" using constructed standard curves.

**POPULATION STRUCTURE IN THE EASTERN OYSTER *CRASSOSTREA VIRGINICA* ASSESSED BY SINGLE NUCLEOTIDE POLYMORPHISMS.** Robin L. Varney and Patrick Gaffney, University of Delaware, Smith Lab, 700 Pilot-town Rd., Lewes, DE 19958.

Single nucleotide polymorphisms (SNPs) are abundantly distributed throughout plant and animal genomes. Because the number of loci is virtually unlimited and they are amenable to robust high-throughput genotyping, SNPs are rapidly becoming standard tools for the analysis of population structure, genetic mapping, and taxonomic identification. Our goal is to evaluate population structure of the eastern oyster throughout its range from Atlantic Canada to the Yucatan Peninsula, using a large number of SNPs identified in known genes (Type I SNPs). While previous work has shown a clear genetic break between Atlantic and Gulf coast populations in mitochondrial DNA, studies of geographic variation in nuclear loci (allozymes and anonymous DNA markers) have yielded mixed results. Existing genomic and expressed sequence tag (EST) databases of *C. virginica* have been mined for the development of both anonymous and type I markers, with recent efforts focused on genes thought to be involved in disease response. Direct sequencing of amplified targets has revealed extensive polymorphism in *C. virginica*. We have developed a set of nuclear SNP markers to examine the genetic diversity of *C. virginica* throughout the species range and provide multiple markers for genetic linkage mapping. Significant heterogeneity in allelic frequencies among the geographic locations was observed for each locus. Our results are consistent with the Gulf-Atlantic split previously observed with other markers. In addition, clinal patterns in allelic frequencies in Atlantic populations are suggested for some loci.

**ASSESSING IMPACTS OF SHELLFISH AQUACULTURE ON EELGRASS POPULATIONS IN EASTERN LONG ISLAND SOUND.** Jamie Vaudrey<sup>1</sup>, Tessa Getchis<sup>2</sup> and Bob Britton<sup>2</sup>. <sup>1</sup>University of Connecticut, Department of Marine Sciences, 1080 Shennecossett Road, Groton, CT 06340. <sup>2</sup> University of Connecticut, Connecticut SeaGrant, 1080 Shennecossett Road, Groton, CT 06340.

Eelgrass (*Zostera marina*) is the dominant vascular plant of northern estuaries of the east and west coasts of the United States. Eelgrass beds provide critical ecological functions such as removing nutrients and stabilizing fine sediments. Beds also provide critical habitat to a myriad of marine organisms including juvenile fish, shellfish, and crustaceans, among others. Eelgrass is on the decline in most estuaries, coincident with urbanization and the resulting increase in nutrient loads to the coastal waters. The presence of eelgrass is considered an indicator of a "healthy" system and much attention has been focused on investigating potential causes of the loss of eelgrass and determining methods to minimize the decline in eelgrass abundance. Bivalve aquaculture, specifically the utilization of submerged cultivation and depuration



gear such as cages, has been implicated as a potential source of negative impacts to eelgrass populations. However, shellfish aquaculture gear has also been shown to provide an equivalent or greater degree of ecosystem services as submerged aquatic vegetation such as eelgrass. This study was conducted to determine the type and degree of impacts and benefits that oyster depuration bottom cages have on eelgrass and surrounding water and sediment quality, in order to provide producers and policy-makers with the scientific data needed to make informed decisions regarding the siting of aquaculture in Long Island Sound. Preliminary results suggest an increase in eelgrass growth rate, measured as sheath length. No treatment effect was seen for water column properties, sediment % organics, or benthic microalgae concentrations.

**FRESHWATER MUSSELS: SPECIES ROLES, ECOSYSTEM SERVICES AND CONSERVATION.** Caryn C. Vaughn, Daniel E. Spooner and Heather S. Galbraith. Oklahoma Biological Survey and Department of Zoology, University of Oklahoma, Norman, OK 73019.

Our lab is using an integrative approach of comparative field observations, field experiments, and laboratory experiments to address three fundamental questions concerning freshwater mussel guild: (1) What is the importance of the mussel guild to stream ecosystem function?; (2) Do species perform different ecological roles?; and (3) Do species roles vary with environmental context? Our results indicate that many ecosystem services performed by mussels (algal clearance, nutrient excretion, biodeposition) are linearly related to community biomass; thus, there is the potential for strong ecosystem effects when mussel biomass is high and hydrologic residence times are long. Algal growth is higher on the shells of living mussels compared to shells alone, and macroinvertebrate richness and densities are higher on the shells of living mussels and in mussel patches than in other streambed areas. Field and laboratory experiments demonstrate strong effects of a potential driver species, *Actinonaias ligamentina*, on factors such as periphyton biomass, but comparatively weak diversity effects. In addition, species effects are context-dependent and regulated by abiotic factors such as temperature and flow. Our results indicate that some mussel species are performing differently in streams and are thus not redundant, but that performance and potential redundancy are context dependent. Using an extensive dataset on mussel community composition and biomass from across southeastern Oklahoma and western Arkansas, we are scaling up our results to make predictions about mussel contributions to ecosystem services in natural streams and how this may vary as the environment changes.

**MANAGING A DUNGENESS CRAB (*CANCER MAGISTER*) FISHERY FOR MULTIPLE USER GROUPS: THE PUGET SOUND, WASHINGTON, EXPERIENCE.** Lisa Veneroso and Richard Childers. Washington Department of Fish and Wildlife.

Intensive fishery management strategies have been adopted for Dungeness crab (*Cancer magister*) in Puget Sound, Washington, in response to increases in fishing pressure. In 1994, a U. S. Dis-

trict Court decision ruled treaty Indian tribes in Washington State have the legal right to harvest up to 50% of shellfish, including Dungeness crab, in their usual and accustomed fishing grounds. Prior to 1995, Dungeness crab were harvested exclusively by a state commercial and recreational fishery using a passive "3S" harvest strategy of minimum size, set season, and sex (male crab only). The guiding assumption of the 3S strategy being that male crab > 6 1/4 inches are surplus and can be harvested at a high rate and not threaten reproductive success or the sustainability of the resource. Since 1995, crab harvest has increased from three million to eight million pounds, harvest seasons have been adjusted on a regional basis to more closely adhere to molt timing, and annual pre-season quotas have been established. Currently, the fishery is managed through harvest sharing agreements that allocate harvest between 15 treaty tribes conducting predominantly commercial fisheries, a state commercial fishery consisting of 250 licenses and a recreational fishery with an estimated 200,000 anglers. Fishery managers are questioning whether the management scheme adopted for Puget Sound is sustainable considering the high exploitation rate, the handling mortality of female and sub-legal size male, and the lack of stock abundance information needed to evaluate the appropriateness of the pre-season quotas.

**ENERGY STORAGE AND UTILIZATION IN RELATION TO GAMETOGENESIS OF *ARGOPECTEN VENTRICOSUS*.** Janel Rogelio Villalaz Guerra, Juan A. Gomez Herrera and Luis D'Croz. Departamento de Biología Marina, University of Panama.

A laboratory study was carried out to observe changes in reproduction of *Argopecten ventricosus* by using histological techniques in gonads and chemical analysis of digestive gland, adductor muscle, mantle-gills and gonad. These data allowed us to calculate energy storage related to gametogenesis of *A. ventricosus*. During 66 days, combinations of monocultures (50:50) of C-ISO and CH-I were added daily to a tank with filtered and aerated seawater. Salinity and temperature of the water were measured with a salinity-temperature probe meter (YSI). Phytoplankton densities were recorded by direct count with a hemacytometer. This study is a contribution to the reproductive biology of *A. ventricosus* and fisheries management of the tropical scallop.

**DIFFERENT TYPES OF NEOPLASIA IN *CRASSOSTREA GIGAS*, *VENUS VERRUCOSA* AND *MYTILUS GALLOPROMINIALIS* FROM THE SPANISH COAST.** Antonio Villalba, Centro de Investigaciones Mariñas, Xunta de Galicia, P.O. Box 13, 36620 Vilanova de Arousa, Spain.

New cases of neoplasia corresponding to three types of neoplastic condition are described. One out of 22 oysters, *C. gigas* taken from an oyster farming area in the Ría de Arousa (Galicia,



NW Spain) showed disseminated neoplasia (DN). Histological analysis revealed abundant abnormal cells with large (up to 5  $\mu\text{m}$ ) round nucleus and scant cytoplasm infiltrating the connective tissue close to the stomach and in blood sinuses, suggesting invasiveness; mitotic figures were frequent but other organs appeared free of abnormal cells, suggesting an early stage of disease. DN prevalence in oysters *Ostrea edulis* cultured in that area is high, thus the possibility of interspecies transmission should be assessed. One out of 25 warty clams *Venus verrucosa* taken from a bed close to Maó (Balearic Islands, Spain) appeared affected by another type of neoplasia. Histological analysis revealed abnormal cells with large, extremely elongated (up to 10  $\mu\text{m}$  long) nucleus and scant cytoplasm replacing normal cells of the connective tissue of the visceral mass; mitotic figures were frequent and no abnormal cell was observed in blood vessels. One out of thousands of mussels *Mytilus galloprovincialis* examined in a histopathological programme ran for 10 years throughout the Galician Rías were affected by germinoma. The mussel was hermaphrodite, with some gonad follicles containing either male or female gametes and some other follicles appeared filled with highly basophilic cells, tightly packed, likely deriving from germinal epithelium of the follicles. Mitotic figures were seen in those masses but they were not frequent.

#### COMPARISON OF IMMUNE PARAMETERS BETWEEN OYSTER STOCKS AND SPECIES WITH DIFFERENT SUSCEPTIBILITY TO INFECTION BY *BONAMIA OSTREAE*.

Antonio Villalba, Pilar Comesaña, Sandra M Casas and Asunción Cao. Centro de Investigacións Mariñas, Xunta de Galicia, Spain.

The parasitic protozoan, *Bonamia ostreae*, causes oyster, *Ostrea edulis*, mass mortalities throughout the European Atlantic coast, whereas the allochthonous oyster, *Crassostrea gigas*, is resistant to bonamiosis. Previous studies indicated that the *O. edulis* strain "Rossmore" produced through selective breeding has increased tolerance to bonamiosis compared to other European stocks of flat oyster. The immune capability of oyster *O. edulis* stocks with different susceptibility to bonamiosis and that of oysters *C. gigas* were compared to assess whether some immune parameters could explain the differences in susceptibility to infection by *B. ostreae* between oyster stocks/species. The *O. edulis* stocks used in the comparisons were: Rossmore (selected strain), oysters from Tralee Bay (Ireland) where the parasite had not previously been detected, and oysters from natural beds in Galician Rías (Spain) that are affected by bonamiosis. The immune parameters selected for comparisons were total haemocyte count and differential haemocyte count of the haemolymph; phagocytic ability of haemocytes; intra- and extracellular production of superoxide anion, production of hydrogen peroxide and production of nitric oxide by haemocytes; antibacterial activity in haemocytes and plasma; phenoloxidase and acid phosphatase activities in haemocytes and plasma; and protease inhibitory activity in plasma.

Consistent differences in any of the immune parameters studied between *O. edulis* stocks that explained differences in susceptibility to bonamiosis were not found. However, some of the immune parameters would be candidates to explain differences in susceptibility to bonamiosis between *O. edulis* and *C. gigas*.

#### STOCK-RECRUIT RELATIONSHIPS FOR *CRASSOSTREA VIRGINICA* AND *C. ARIAKENSIS* IN CHESAPEAKE BAY DEVELOPED FOR A DEMOGRAPHIC OYSTER POPULATION MODEL. Jon H. Volstad and Jodi R. Dew, Versar, Inc., 9200 Rumsey Road, Columbia, MD 21045.

A demographic population model is being developed to support an ecological risk assessment (ERA) of the proposed introduction of *Crassostrea ariakensis* and restoration alternatives of *C. virginica* in Chesapeake Bay by the states of Maryland and Virginia. As part of the model, a stock-recruit relationship was developed and is defined as the ratio of number of spat recruited to October (when the annual Fall survey is performed by the Maryland Department of Natural Resources (DNR)) to number of standardized (to 77 mm) female oysters. Stock-recruit relationships were estimated using empirical data collected from 1980 to 2004 by the DNR in annual surveys of oyster beds in Maryland, shell length data, sex ratio by size estimates, and fecundity relationships between shell length and number of eggs produced. R-square value for *C. virginica* stock-recruit relationship was 0.45 with 2.6 spat produced per standardized female oyster. The number of spat produced was 2.6, 2.8, and 2.1 per standardized female oyster when the stock-recruit relationship was examined per average, dry, and wet rainfall year, respectively. This stock-recruit relationship for *C. virginica* is used for *C. ariakensis* to predict the number of spat produced per standardized *C. ariakensis* stock to a 77mm female *C. virginica* oysters, under the assumption of similar mortality rates for spat of both species to October. The stock-recruit relationship for both species can also be altered to include gamete loss from inviable hybrids in cross-fertilization when species are reproducing in close proximity to each other.

#### ESTIMATION OF ANNUAL MORTALITY RATES FOR EASTERN OYSTERS (*CRASSOSTREA VIRGINICA*) IN CHESAPEAKE BAY BASED ON BOX COUNTS AND APPLICATION OF THOSE RATES FOR POPULATION GROWTH PROJECTIONS. Jon H. Volstad<sup>1</sup>, Jodi R. Dew<sup>1</sup> and Mitchell Tarnowski<sup>2</sup>. <sup>1</sup>Versar, Inc., 9200 Rumsey Rd., Columbia, MD 21045. <sup>2</sup>Maryland Department of Natural Resources.

In an effort to restore the ecological role of oysters in Chesapeake Bay and the economic benefits of a commercial fishery, the states of Maryland and Virginia are considering the introduction of

the non-native Asian oyster, *Crassostrea ariakensis*. As part of the ecological risk assessment (ERA) to evaluate the proposed action and restoration alternatives, demographic modeling is applied to project the change in populations of both Asian and eastern oyster populations in the Bay in space and time. We present two approaches to parameterize the annual mortality rates for the Asian and eastern oyster for the demographic model. Mortality rates were estimated from empirical data collected by the Maryland Department of Natural Resources (DNR) in annual surveys of oyster beds in Maryland. We compared counts of recent boxes (dead oysters including gapers about two weeks old, in which tissue is still found within the shell, as well as boxes with no fouling or sedimentation on the inner valve surfaces), old boxes (dead oysters in which fouling and/or sedimentation is found on the inner valve surfaces and no tissue remains), and live oysters in both market and small size classes. Our mortality estimates from recent box counts consistently differentiated between years with high versus low disease intensity and wet and dry years, and also between salinity zones. In contrast, traditional estimates of yearly mortality based on total box counts were often out of phase with measured disease intensity levels, and type of weather year (dry or wet).

**UNDERSTANDING DIET DEVELOPMENT IN RELATIONSHIP TO SEA URCHIN GAMETOGENESIS.** Charles W. Walker and S. Anne Boettger, The University of New Hampshire.

One challenge when culturing adult green sea urchins is the formulation of diets yielding palatable and marketable roe. Current commercial diet formulations vary and yield taste, color and texture variation of the product. High protein content of diets related to production of large gonads yields poor taste, while color is related to carotenoid content. Nutrient uptake into nutritive phagocytes (NPs) of the gonad (somatic cells in both sexes) is an important factor as sea urchin gonads are large before and during gametogenesis due to nutrient storage by NPs. Initially, NPs incorporate nutrients into membrane-bound vesicles in the cytoplasm and mobilize them as germ cells enlarge during gametogenesis. Mechanisms of nutrient delivery to NPs and from them to germ cells have been a focus of recent studies. At completion of oogenesis NPs contain basal amitotic oogonia and small/intermediate-sized vitellogenic primary oocytes, though their major activity is enclosure and nourishment of vitellogenic oocytes prior to maturation and release. Mechanisms regulating differential nutrient release by sea urchin NPs and their selective uptake by germ cells at different stages of gametogenesis are unknown though there is coordination between NPs and germ cells. Oocytes must be receptive to incorporate nutrients released by NPs at specific times. Endocytosis by oocytes increases two fold, uptake of MYP increases 10-fold during this time. It is inevitable that changes in nutrients delivered to germ cells may be related to the

quantity/quality or kind of food ingested by sea urchins, which has to be taken into account during diet development.

**PHYSIOLOGICAL RESPONSES TO SALINITY STRESS IN THE FLATBACK MUD CRAB *EURYPANOPEUS DEPRESSUS*.** Laura D. Walls and S. Gregory Tolley, Florida Gulf Coast University, Coastal Watershed Institute, 10501 FGCU Blvd. South, Ft. Myers, FL 33965.

Estuaries in Southwest Florida experience highly altered freshwater inflow resulting in part from anthropogenic activities. To gauge possible effects of altered salinity regimes on species in these systems, physiological responses to salinity stress were investigated in the flatback mud crab, *Eurypanopeus depressus*, a dominant member of oyster-reef communities in the region. Haemolymph osmolality and oxygen consumption were measured for animals acclimated to 5‰, 15‰, 25‰, and 35‰ over a period of four weeks in a laboratory setting. Haemolymph osmolality varied significantly with respect to salinity, with measured osmolalities at each salinity treatment being significantly different ( $p < 0.0001$ ) from one another. Oxygen consumption also varied significantly with salinity, with values recorded for the lowest salinity treatment (5‰) being generally higher ( $p < 0.0001$ ) than all other treatments over the length of the study. Oxygen consumption did not vary over the first three weeks of the study for animals acclimated to 5‰ but declined significantly by week four. This general pattern of increased oxygen consumption in response to dilute salinity levels is a trend that has been documented previously in several estuarine species. The results of this study suggest that osmoregulation requires greater energy expenditure by *E. depressus* at sub-normal salinity levels. Having to expend additional energy to meet osmoregulatory demands can result in reduced amounts of energy available for processes such as growth and reproduction, thereby affecting organism health. Suboptimal salinity levels resulting from altered freshwater inflow can thus significantly affect species abundance and distribution, specifically in the upper reaches of estuaries.

**IMPACT OF WATER MOTION ASSOCIATED WITH RECREATIONAL BOATING AND HURRICANES ON INTERTIDAL OYSTER REEFS.** Linda Walters<sup>1</sup>, Paul Sacks<sup>1</sup>, Loren Coen<sup>2</sup>, M. Yvonne Bobo<sup>3</sup>, Donnia Richardson<sup>3</sup>, Melinda Donnelly<sup>4</sup>, Sarah Johnson<sup>4</sup> and Heidi Deutsch<sup>4</sup>. <sup>1</sup>University of Central Florida, <sup>2</sup>South Carolina Department of Natural Resources, <sup>3</sup>South Carolina Department of Natural Resources, <sup>4</sup>University of Central Florida.

In major recreational boating channels in shallow-water estuaries along the east coast of Florida, piles of disarticulated shells that extend up to one m above the high tide line on the seaward

edges of intertidal reefs of *Crassostrea virginica* are becoming more and more abundant. As part of a larger study to understand the impact of these “dead margins” on critical habitats, we addressed the following questions: 1) what is the relative importance of boat wakes versus larger-scale, naturally generated (e.g. hurricane) wakes in shaping reef profiles, 2) what is the prevalence and intensity of oysters with *Perkinsus marinus* on pristine reefs versus reefs with dead margins, and 3) what is the potential for dead margins to be colonized by native and invasive plants. We documented significant shell movement from a single boat pass by a reef, while 22 m/s winds moved less than 1% of deployed shells. Additionally, the three hurricanes in 2004 impacting this area had negligible effects on reef profiles. Likewise, no significant differences were detected for *Perkinsus* in oysters between pre- and post hurricane sampling on dead or healthy reef margins. Red mangroves produce seeds year-round in central Florida and these seeds colonized dead margins if they recruited between winter and mid-summer. Water levels were higher each fall, preventing recruitment of red or late-summer black mangrove seeds at this time. Invasive Brazilian pepper seeds dispersed each winter. Although these seeds were frequently observed on dead margins, none were retained long enough for recruitment to occur.

**TO PIT OR NOT TO PIT?: COMPARISON OF OVERWINTERING METHODS FOR OYSTERS.** William C. Walton<sup>1</sup>, Ben Wright<sup>2</sup> and Diane C. Murphy<sup>3</sup>. <sup>1</sup>Cape Cod Cooperative Extension & Woods Hole Sea Grant. <sup>2</sup>Cape Cod AmeriCorps, <sup>3</sup>Cape Cod Cooperative Extension.

The threat of ice damage has forced intertidal oyster farmers to devise strategies to reduce potential losses. In New England, some oyster farmers move their oysters from the farm location to storage area, or ‘seed pit’, that is typically cold (0–4°C) and damp (over 90% relative humidity). Qualitatively, local farmers have reported survival rates of over 90%. We quantified survival and typical time to revival of oyster, (*Crassostrea virginica*) seed as a function of storage time. While survival was good and did not seem to vary over the course of the experiment, there was a clear step-wise drop in time to revival. Furthermore, a study of survival of seed held in the pits of local farmers suggested survival remained high (≥86%) for seed held for ~4 months but dropped to 82% for those held an additional month. Furthermore, evidence suggests that second year seed do not survive pitting as well as first year seed. Alternatives to pitting include leaving the oysters on the farm location or moving them to deeper water, below any ice that might form. We compared overwintering survival and growth of these three methods, both immediately post-winter and across the 2005 growing season. The lowest survival was in the treatment left on the flats, apparently due to damage from ice, while little to no growth was

observed immediately post-winter. More surprisingly, there was a tendency for growth across the season to be slowest in the treatment moved to deeper water, relative to the other two treatments.

**IDENTIFICATION OF CRASSOSTREA SPECIES FROM CHINA USING SNP-BASED MARKERS.** Haiyan Wang and Ximing Guo, Haskin Shellfish Research Laboratory, Rutgers University.

China is home to 17 species of oysters and among them, five *Crassostrea* species: *C. hongkongensis*, *C. angulata*, *C. gigas*, *C. sikamea* and *C. ariakensis* are most common and commercially important. These five species often co-exist in the same estuary, and their identification using morphological characteristics is problematic. Genetic markers are needed for rapid and reliable identification of these oysters. Single nucleotide polymorphisms (SNPs) are simple and powerful markers for various genetic analyses. In this study, we developed species-specific SNP markers for the identification of common oysters from China. The mitochondrial cytochrome oxidase I (COI) gene and the nuclear 28S ribosomal RNA gene were used for marker development. DNA sequences from different species were either obtained by direct sequencing or downloaded from GenBank. Sequences were aligned, and species- and genus-specific SNPs were identified. Primers were designed for species/allele-specific amplification to generate fragments of different sizes in each species. A multiplex set of species-specific markers from COI was able to distinguish all five *Crassostrea* species in a single-tube PCR. It also separated *Ostrea* and *Saccostrea* species from *Crassostrea* species with the exception of *C. virginica* and *C. rhizophorae*. The 28S primer set was able to separate *C. hongkongensis*, *C. ariakensis* from other species, as well as *Saccostrea* and *Ostrea* species from *Crassostrea* species (except *C. virginica* and *C. rhizophorae*). The SNP-based markers do not require fluorescence-labeling or post-PCR digestion, providing a simple, fast and reliable method for oyster identification.

**DISTRIBUTION OF CRASSOSTREA ARIAKENSIS IN CHINA.** Haiyan Wang<sup>1</sup>, Lumin Qian<sup>2</sup>, Guofan Zhang<sup>3</sup>, Xiao Liu<sup>4</sup>, Aimin Wang<sup>4</sup>, Yaohua Shi<sup>4</sup>, Nianzhi Jiao<sup>5</sup> and Ximing Guo<sup>1</sup>. <sup>1</sup>Haskin Shellfish Research Laboratory, Rutgers University, <sup>2</sup>Third Institute of Oceanology, Oceanic Administration, PRC, <sup>3</sup>Institute of Oceanology, Chinese Academy of Sciences, PRC, <sup>4</sup>Ocean College, Hainan University, <sup>5</sup>Center for Marine Environmental Sciences, Xiamen University.

It is commonly assumed that *Crassostrea ariakensis* is synonymous with *C. rivularis* that, according to the literature, is abundant and widely distributed in China. However, at least three species, *C.*

*gigas*, *C. hongkongensis* and *C. ariakensis*, have been reported as *C. rivularis* in China, creating uncertainties about the distribution of *C. ariakensis*. To determine the distribution of true *C. ariakensis* in China, we collected and classified 2,624 oysters from 50 locations along China's coast using species-specific DNA markers. *C. ariakensis* was found at 10 sites ranging from northern Shandong to Guangxi. While *C. ariakensis* had a wide geographical distribution, its occurrence within its range is patchy or scarce. Overall, *C. ariakensis* accounted for only 9.5% of all oysters collected. Large *C. ariakensis* populations were found in only three areas: Jiulong River in Fujian, Dongzao Harbor near Yangtze River and Yellow River basin in Bohai Sea, with none observed in-between. In Guangxi and Guangdong, *C. ariakensis* was present in all samples collected at low frequencies (0.5–17.5%). All three major populations are found in or near large rivers, and the absence of rivers may be a factor contributing to the fragmented distribution. At all sites, *C. ariakensis* co-existed with other species: *C. gigas* in Bohai Sea; *C. sikamea* at Dongzao Harbor; and *C. hongkongensis*, *C. angulata*, *C. sikamea* and *Saccostrea* species in southern China. *C. ariakensis* tended to occur subtidally, while other species were often found intertidally.

**LOSS OF ALLELE DIVERSITY IN AN INTRODUCED POPULATION OF THE HERMAPHRODITIC BAY SCALLOP *ARGOPECTEN IRRADIANS*.** Lingling Wang<sup>1</sup>, Huan Zhang<sup>2</sup>, Linsheng Song<sup>2</sup> and Ximing Guo<sup>1</sup>. <sup>1</sup>Haskin Shellfish Research Laboratory, Rutgers, US, <sup>2</sup>Institute of Oceanology, CAS, China.

The bay scallop, *Argopecten irradians*, a hermaphroditic bivalve native to the Atlantic coast of the United States, was introduced to China for aquaculture production in 1982. Now bay scallops dominate scallop farming in China and account for more than half of all scallop production. Bay scallop aquaculture is exclusively hatchery based and as the initial introduction consisted of only 27 scallops, there have been concerns about inbreeding and inbreeding depression in cultured populations in China. In this study, we used 11 simple sequence repeat (SSR) markers to compare genetic variation in cultured populations from China with that in a natural population from America. Eight of the 11 loci were polymorphic in all populations studied. Although the difference in heterozygosity was small, the Chinese populations lost 10 of the 45 alleles (22%) found in the wild population. The reduced allele diversity suggests that the Chinese populations experienced a severe bottleneck in genetic diversity during the introduction and subsequent hatchery production. Interestingly, the loss of rare alleles did not result in significant changes in heterozygosity. Fixation index of the Chinese populations were actually lower than that of the wild population, suggesting that the culture Chinese populations may represent a mixture of scallops from different introductions or populations. Effects of the reduced allele diversity are not clear at this time, but results of this study highlight the need for

genetic monitoring and selective breeding in order to ensure the genetic health of introduced populations.

**DEVELOPMENT AND CHARACTERIZATION OF EST-SSR MARKERS IN THE EASTERN OYSTER *CRASSOSTREA VIRGINICA*.** Yongping Wang and Ximing Guo, Rutgers University.

Simple sequence repeat (SSR) markers were developed from expressed sequence tags (ESTs) in the eastern oyster (*Crassostrea virginica*). ESTs of the eastern oyster were downloaded from GenBank and screened for SSRs that contained at least eight units of di-nucleotide repeats or five units of tri-, tetra-, penta- and hexa-nucleotide repeats. The screening of 9101 ESTs identified 127 (1.4%) SSR-containing sequences. Primers were designed for 88 SSR-containing ESTs with good and sufficient flanking sequences. PCR amplification was successful for 71 (81%) of the primer pairs including 19 pairs amplified fragments that were significantly longer than the expected size, probably due to introns. Sixty-six pairs that produced fragments size shorter than 800 bp were screened for polymorphism in five oysters from three populations using polyacrylamide gels, and 53 of them (80%) were polymorphic. Forty-five SSRs were labeled and genotyped in 30 oysters from three populations using an automated sequencer. Five of the SSRs amplified more than two fragments per oyster, suggesting that they belonged to duplicated loci, but they can still be used for segregation analysis. The remaining 40 SSRs had two alleles per individual including seven with null-alleles. In the 30 oysters analyzed, the SSRs had an average of 9.6 alleles per locus, ranging from 2 to 24. All 45 markers were used for segregation analysis in a family with 34 progeny. None of the 45 loci showed significant deviation from Mendelian ratios. This study demonstrates that ESTs are valuable resources for the development of genetic markers in the eastern oyster.

**IDENTIFICATION OF *CRASSOSTREA ARIAKENSIS* USING ITS LENGTH POLYMORPHISM.** Yongping Wang and Ximing Guo, Haskin Shellfish Research Laboratory, Institute of Marine.

Oysters cannot be reliably identified using morphological characteristics alone. In an effort to develop genetic markers for oyster identification, we studied length polymorphism in internal transcribed spacers (ITS) between ribosomal RNA genes in 12 common species of Ostreidae: *Crassostrea virginica*, *C. rhizophorae*, *C. gigas*, *C. angulata*, *C. sikamea*, *C. ariakensis*, *C. hongkongensis*, *Saccostrea echinata*, *S. glomerata*, *Ostrea angasi*, *O. edulis*, and *O. conchaphila*. We downloaded and aligned ribosomal RNA sequences from all oyster and some other bivalve species to identify conserved sequences flanking ITS1 and ITS2. We designed two pairs of primers and optimized PCR conditions for simultaneously amplification of ITS1 and ITS2 in a single tube. Amplification was successful in all 12 species, and PCR products were

visualized on high-resolution agarose gels. ITS2 was longer than ITS1 in all *Crassostrea* and *Saccostrea* species, while they were about the same size in three *Ostrea* species. No intraspecific variation in ITS length was detected. Among species, the length of ITS1 and ITS2 was polymorphic and provided unique identification of eight species or species pairs: *C. ariakensis*, *C. hongkongensis*, *C. sikamea*, *O. conchaphila*, *C. virginica*/*C. rhizophorae*, *C. gigas*/*C. angulata*, *S. echinata*/*S. glomerata*, and *O. angasi*/*O. edulis*. Two species within a pair were not distinguishable by ITS length. The ITS assay provides simple, rapid and effective identification of *C. ariakensis* and several other oyster species. Because the primer sequences are conserved, the ITS assay may be useful in the identification of other bivalve species.

**NUTRITION RESEARCH IN ECHINODS: CONSIDERATIONS FOR METHODOLOGY.** Stephen A. Watts<sup>1</sup>, M. L. Powell<sup>1</sup>, A. L. Lawrence<sup>2</sup> and J. M. Lawrence<sup>3</sup>. <sup>1</sup>University of Alabama at Birmingham, <sup>2</sup>Texas A&M University System, <sup>3</sup>University of South Florida.

Recent interest in commercial echinoid culture has led to studies of the nutritional requirements for many sea urchin species. As sea urchins are benthic, eurythermal, stenohaline and, in some cases, rheophilic organisms, which feed at relatively slow or irregular rates, consideration of abiotic and biotic factors that affect feeding and growth characteristics can enhance our ability to evaluate nutritional requirements. We are attempting to standardize protocols for nutritional studies in echinoids. Considerations include standardized temperatures (species-specific), salinity, photoperiod, oxygen levels, water quality (including nitrogen levels, pH, hardness and alkalinity, natural vs. artificial seawater), flow rates, and *in situ* bacterial and fungal populations. System design (flow-through or recirculation) must be coupled with appropriate statistical design and evaluation (individual observations vs. group trials). Understanding life-history traits, including behaviors, pathologies, and natural growth variability, is essential. Feeds can include live or preserved natural food and practical, semi-purified and purified feeds. However, evaluation of natural food is of limited value in determining requirements because of lack of information about their content. Formulated (*not* artificial) feeds should be composed of quality ingredients available from commercial sources, whether practical, semi-purified or purified. Formulated feeds must have defined size, shape, texture, stability, and attractability, with defined handling and storage characteristics. Whenever possible, nutrient content should be determined empirically. Feed management strategies must be well defined, including feeding rates and rations. Finally, echinoid nutritional studies should use standardized metrics and responses for cross-species and cross-phylum comparisons.

**DEVELOPMENT OF FORMULATED FEEDS IN ECHINODS.** Stephen A. Watts<sup>1</sup>, A. L. Lawrence<sup>2</sup>, M. L. Powell<sup>1</sup> and J. M. Lawrence<sup>3</sup>. <sup>1</sup>University of Alabama at Birmingham, <sup>2</sup>Texas A&M University System, <sup>3</sup>University of South Florida.

Successful sea urchin aquaculture will ultimately depend on high quality, nutritionally-complete, commercially-available feeds. We are developing practical, semi-purified and purified feeds that promote high rates of growth and survival. These feeds require the use of high quality ingredients that can be obtained from reputable commercial vendors. Semi-purified diets can be used to determine dietary requirements and/or toxicities of proximate nutrients in urchins. Purified diets can be used to evaluate requirements for micronutrients, including vitamins and minerals. We are currently evaluating semi-purified, cold-extruded feeds that range from 17 to 41% protein, 15 to 36% carbohydrate, 4 to 12% crude fat (marine and non-marine sources), 1 to 5% crude fiber, and 6 to 50% ash. These nutrients are evaluated using both purified and non-purified sources, both in combination and as the sole source. Amino acid profiles have been calculated to ensure adequate availability of indispensable amino acids. Fatty acid profiles have been determined to evaluate requirements for PUFA or HUFA. Data indicate that specific minerals and vitamins are required for normal physiological function. High production of gonads with marketable color and flavor is now possible. Empirically-derived levels of experimental nutrients are necessary as calculated levels may inaccurately estimate actual levels in the feeds.

**GROWTH OF BAY SCALLOPS, *ARGOPECTEN IRRADIANS IRRADIANS*, FED VARYING *TETRASELMIS CHUI* CONCENTRATIONS AND ITS EFFECT ON AMMONIA CONCENTRATION.** James C. Widman Jr., Shannon Meseck, David J. Veilleux and Mark Dixon. USDOC, NOAA, National Marine Fisheries Service Milford CT.

Bay scallops, *Argopecten irradians irradians*, were grown in semi-static culture systems containing *Tetraselmis chui* at concentrations of 1,000, 3,500, 8,000 and 11,000 cells/ml. Our objective was to determine whether phytoplankton concentrations would affect ammonia levels in the culture system. Microalgal cell concentrations were continuously monitored and maintained by a computer control system. F media was used to grow the *T. chui*. Daily buckets were drained and filled with 15 L of filtered seawater. Twice weekly scallops were removed from the containers and placed in clean 15 L buckets. Every week ammonia levels were measured during a 24 hour period. Scallops grew from initial mean shell heights of 10.5–10.9 mm to final mean shell heights of 15.2–17.9 mm. Scallops grew at similar rates for the first 14 days. After 21 days scallops grown at a cell concentration of 11,000 cells/ml were significantly smaller than those grown at 1000 cells/ml. Throughout the experiment scallops grown at 1,000 and 3,500 cells/ml were not significantly different ( $p > 0.05$ ) from one another. Similarly scallops grown at 3,500 and 8,000 cells/ml grew at

similar rates ( $p > 0.05$ ). Scallops grown at 8,000 and 11,000 cells/ml were always similar ( $p > 0.05$ ) to one another. These results contrast with a previous study that had shown no growth differences when scallops were fed varying concentrations of *T. chui* grown in E media. Ammonia concentrations were never measured above 10  $\mu\text{mol/L}$ . There was no relationship between ammonia concentrations observed and scallop growth rates. Scallop survival was greater than 83% in all treatments.

**OYSTER AQUACULTURE MAY POSITIVELY AFFECT EELGRASS (*ZOSTERA MARINA* L.) THROUGH ENHANCED SEED PRODUCTION AND GERMINATION.**

**Lorena M. Wisheart<sup>1</sup>, Sally D. Hacker<sup>1</sup>, Brett R. Dumbauld<sup>2</sup> and Jennifer L. Ruesink<sup>3</sup>.** <sup>1</sup>Oregon State University, Zoology Department, Cordley 3029, Corvallis, OR 97331-2914, <sup>2</sup>USDA/ARS, <sup>3</sup>University of Washington.

The interactions between oyster aquaculture and eelgrass need to be explored to assist shellfish growers in the development of sustainable farms while ensuring ecological integrity in aquaculture areas. Past studies have identified both positive and negative effects of shellfish aquaculture on eelgrass but researchers have yet to address how such activities may affect eelgrass recruitment. We conducted surveys in Willapa Bay, WA and found higher seedling densities in dredged beds than in longlines or eelgrass beds. We hypothesized that this pattern was due to variations in seed density and/or differences in germination. We estimated seed density by counting the number of seeds produced per shoot in each habitat type and found this to be highest in dredged beds and lowest in longlines. We also tested the hypothesis that dredging positively influences germination by adding seeds to each of the three habitat types in areas with eelgrass present and adjacent plots where adult plants were removed. Germination was highest in the eelgrass beds, where, interestingly, eelgrass removal had a positive effect. We found a similar result when seeds were added to control and removal plots at five different eelgrass beds throughout the bay. Higher germination in removal plots suggests that reduced competition for light and other resources may positively influence recruitment. Greater recruitment in dredged beds may thus be due to both enhanced seed densities as well as removal of neighboring adults. Together these studies suggest ground culture practices may positively affect eelgrass recruitment while longlines may have a negative effect.

**GENETIC DIFFERENTIATION AMONG FOUR *CRASSOSTREA ARIAKENSIS* POPULATIONS IN ASIA BY MICRO-SATELLITE POLYMORPHISM.** Jie Xiao, Jan F. Cordes and Kimberly S. Reece. Virginia Institute of Marine Science, 1208 Greates Road, Gloucester Point, VA 23062.

*Crassostrea ariakensis* is being considered for introduction into Chesapeake Bay to help revive the declining native oyster industry and bolster the local ecosystem. Little is known, however, about

wild populations of *C. ariakensis* in its native region, including native distributions, overall genetic diversity, genetic structure, and levels of gene flow. Several discontinuous natural populations have been identified along a wide geographic range in the western Pacific. It is still unclear whether these all are natural populations or whether some are a result of anthropogenic transportations. We studied the genetic variance among four wild populations of *C. ariakensis* from Ariake Bay, Japan, Kahwa River, South Korea, the Yellow River basin in northern China, and Beihai in southern China, using novel microsatellite makers. Initial results are reported for 3 loci (CarG110, CarI19-6a, CarI1-70) developed from *C. ariakensis* partial genomic libraries and screened for the presence of null alleles in eight family crosses. Estimated multi-locus *F<sub>st</sub>* (0.0168) values were highly significant for all samples, indicating heterogeneous populations exist in these regions. Single locus *F<sub>st</sub>* and *P* values are quite variable among these loci, and additional markers are being developed to further test the null hypothesis of population homogeneity. These preliminary results indicate genetic structure exists among populations of *C. ariakensis* in its native region and suggest that microsatellite markers could serve as efficient genetic tags for monitoring *C. ariakensis* introduced into the Chesapeake Bay from different native gene pools.

**GENETIC VARIATION IN SURVIVAL AND POST-STARVATION GROWTH RECOVERY OF BIVALVE LARVAE (*CRASSOSTREA GIGAS*).** Pauline C. Yu and Donal T. Manahan, University of Southern California, Department of Biological Sciences, Division of Marine Environmental Biology, Los Angeles, CA 90089-0371.

Defining the "point of no return" is of importance for larvae growing in nutrient-poor environments. We tested different larval families of *C. gigas* for their starvation tolerance and ability to recover once fed, following prolonged starvation. Larvae were capable of surviving for over six weeks without food, representing a >6-fold increase over theoretical limitations based on utilization of energy reserves in eggs. Such larvae grew upon feeding at rates comparable to sibling larvae fed at day two. Under our experimental conditions, the "point of no return" for some fraction of a larval cohort far exceeded theoretical expectations. Larvae did not down-regulate metabolism, as respiration rates were maintained at  $\sim 12 \text{ pmol larva}^{-1} \text{ hr}^{-1}$  throughout the period tested for food deprivation. Also, protein content of larvae was conserved in the absence of food with up to 47% of day-2 content being present even after 24 days. Genotypic differences in larval survival and recovery were observed. Eggs from single females showed 2-fold differences in starvation resistance when fertilized with different males, implying important genetic-physiological interactions regarding use of egg energy reserves. When comparing sibling larvae from multiple genetic crosses, size at day 6 for larvae fed from day 2 ("growth heterosis") correlated positively ( $P = 0.02$ ) with time to 50% survivorship for larvae of the same family that were unfed

from day two. We propose that common mechanisms of physiological efficiency might be the bases for growth heterosis under high-food conditions and increased larval survival in the absence of food.

**EFFECTS OF FLUID SHEAR ON RED ABALONE SPERM-EGG INTERACTIONS.** Richard Zimmer and Jeffrey A. Riffell, UCLA.

Fertilization rate is an important factor mediating the production of offspring and, hence, population dynamics. For marine organisms that broadcast their sperm and eggs into the sea, surprisingly little is known about the mechanisms controlling sperm-egg interactions. Here, we investigated hydrodynamic effects on fertilization in red abalone (*Haliotis rufescens*). Experiments were

performed in laminar-shear flows that simulated important aspects of small-scale turbulence within natural habitats. Fertilization rates were determined over a wide range of gamete concentration and shears created in a Taylor-Couette flow tank and using a new application of infrared laser and computer-assisted video imaging technologies. For red abalone, shears of  $< 0.1/s$  maximized sperm chemotaxis, gamete encounter rates, and fertilization success. Fertilization was subsequently well explained by numerical models that took into account cell shapes, sizes, and propulsive forces generated by sperm swimming, as well as shear forces imposed by the flow. These models, thus, established the relative contributions of active sperm behavior and passive physical transport to sexual reproductive processes. Combined findings have value in forecasting field sites that maximize offspring production, for transplants, or outplants, of adult brood stock.





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**COVER PHOTO:** The surfclam, “Macha” (*Mesodesma donacium*) is fished by hand-gathering (scuba-diving in the surf zone or walking the shore line) in Coquimbo, Chile. Photo: Ana Cinti.

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## DEVELOPMENT AND APPLICATION OF TECHNIQUES FOR PREDICTION OF THE SCALLOP *PECTEN MAXIMUS* (L.) SPATFALL

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**ABSTRACT** Over five million spat of the king scallop, *Pecten maximus* (L.) were collected accidentally in the North Water of Mulroy Bay in County Donegal, Ireland in the summer of 1979. This large natural settlement, by far the greatest number obtained at the time in any European trial, led to research investigations in 1980 and 1981 to support the development of this site as a scallop spat collection center. A combination of gonad monitoring, plankton sampling, spat settlement and spat collection trials were used to develop a technique for predicting the date of the scallop spatfall to maximize the yield of scallop spat. A technique for forecasting the date of peak spatfall involving installation of spat collectors when the mean shell length of scallop larvae was 180  $\mu$ m and 50% of the larval cohort exceeded this size was developed. Unlike other sites studied, peak spat collection occurred at 4 m beneath the water surface. Since its development, this technique for prediction of the date of the peak collection has been used commercially, and for illustrative purposes data from 2002 have been provided. The development and application of this forecasting technique has assisted in the collection of millions of scallop spat for ongrowing trials by the scallop culture industry.

**KEY WORDS:** *Pecten maximus*, prediction, scallop settlement, spat collection, spatfall

### INTRODUCTION

Many commercial scallop fisheries currently exploited in European waters are protected against overfishing by legislation applied to fishing gear; in terms of the number, type and specification of dredges; to landings in terms of the quantity and minimum shell length of the catch and to the duration of the fishing season. All of these precautionary measures are designed to prevent existing resources becoming further diminished or potentially exhausted beyond repair and to allow time for the natural recovery of the resource.

Faced with similar restrictive practices, fishermen in Japan adopted a more proactive role towards conservation and implemented measures to expand resources by the transplantation or reseedling of aquatic animals. The development of methods for the mass production of juveniles of selected species in waters suitable for breeding and the subsequent release of these juveniles to the oceans has resulted in dramatically increased yields from several fisheries. Noteworthy in this context is the successful expansion of Japanese scallop cultivation, annual landings increasing from approximately 9,000 t during the 1960s to consistently over 500,000 t since 1995. The success of this approach to scallop fishery management and resource expansion encouraged many European countries to implement scallop research programs during the 1980s with a view to emulating this success.

Early spat collection trials in France, Spain, Scotland, Isle of Man and England showed considerable annual fluctuations in the number of spat per collector and generally disappointing yields compared with results in Japan (Buestel et al. 1976, Pickett 1977, Ventilla 1977a, 1977b, Mason & Drinkwater 1978, Brand et al. 1980, Roman et al. 1985). Scottish trials in which spat collectors were installed at regular intervals over a 4–6 wk period demonstrated the existence of an optimum date for peak spat collection. Temporal variations in the date of the peak spat collection over

five years ranged from late June to early August (Ventilla 1977a, 1977b, Slater, 1978, 1979, 1980). In some years this peak could rise and fall within a one-week period and in other years be extended over a two- to three-week period (Ventilla 1977a, 1977b). Installing spat collectors in the sea either before or after the date of peak spatfall could dramatically reduce the yield of spat by up to 75%. Clearly for natural spat collection to be successful in the provision of an abundant and reliable source of seed for aquaculture and fishery enhancement, there was a need for a spatfall prediction technique similar to that used in Japan.

Despite significant research effort on king scallops since the early 80's commercial scallop cultivation in Europe has not developed and in countries where fishery landings have been expanded, such increases have resulted only from further exploitation of already overfished natural resources. The major limitation to the scale of any scallop farming or stock enhancement program remains as the availability of a consistently large and reliable source of juvenile scallops or spat. Worldwide in those countries where commercial scallop cultivation has developed, for example Japan, China and Chile, problems associated with the provision of a regular supply of scallop spat have first been overcome.

In 1979, a local aquaculture co-operative, the North Water Co-operative Soc. Ltd., during attempts to capture mussel spat, *Mytilus edulis* (L.), accidentally collected on 12-mm diameter polypropylene rope over 5 million scallop spat in the North Water of Mulroy Bay in County Donegal, by far the greatest number obtained at the time in any European trial (Minchin 1980). As a result of this exceptionally large settlement, research trials commenced to support the exploitation of the site as a scallop spat collection center.

Research during 1980 and 1981 focused on gonad monitoring, plankton analysis, spat settlement and spat collection trials to develop a technique for prediction of the date and location of the peak scallop spatfall. This study provides results from these investigations that led to the development of techniques for prediction of the date of peak spatfall and provides for illustrative purposes results obtained during commercial application of this predictive technique in 2002.

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## MATERIALS AND METHODS

### The Location

Mulroy Bay is located in County Donegal in the northwest of Ireland (Fig. 1). It is a sheltered, marine lough connected to the open sea by a series of long and narrow channels. The Bay is well sheltered from excessive exposure and no oceanic swells are able to penetrate into the two main parts, the Broad Water and the North Water. Seawater enters the Bay through the Narrows and flows into the Broad Water before passing through the Moross Channel into a further and even more enclosed sea lough, the North Water, which is approximately 3 km by 1 km in size. Water depths extend to 51 m in the North Water and the bathymetry of the lough is available on Admiralty Chart No. 2698. Resulting from this topography, the tidal range within the Bay and water exchange with the open sea is much diminished. The Bay provides an ideal location for shellfish spat production, because larvae produced by natural populations in the Bay are retained and settle within the system rather than being dispersed as occurs in more open waters.

The sea lough is surrounded by poor quality land with a low population density supporting an agricultural community dominated by small farms. At the head of the lough is the village of Milford (population 1,334) that forms the gateway to both the Fanad Peninsula down the eastern side of Mulroy Bay and the Rosguill Peninsula down the western side of Mulroy Bay. The only significant employers in the area are Marine Harvest (Fanad), a salmon farming company with approximately 150 staff.

### Gonad Monitoring

Samples of adult king scallops for gonad assessment were dredged from around the Stookans, Massmount Bay, North Water of Mulroy Bay using a lightweight converted oyster dredge (Fig. 1). In 1980 samples comprising 22–27 individuals of mean shell height  $113.4 \pm 1.8$  mm were collected at intervals ranging from 4–13 days (mean = 8.4 days) between June 5 and August 30, 1980. In 1981 samples comprising 23–25 individuals of mean shell height  $113.8 \pm 1.2$  mm were collected at intervals ranging from 5–10 days (mean = 6.8 days) between June 10 and July 8, 1981. Soft tissues were dissected from each scallop, pooled and weighed. Gonad index was determined according to the following formula:

$$\text{Gonad Index (\%)} = \frac{\text{Total wt. of gonad (g)}}{\text{Total wt. of soft tissue (g)}} \times \frac{100}{1}$$

To account for minor variations in the size of scallops from week to week, the relative gonad height (RGH) was calculated using the equation:

$$\text{RGH (g.mm}^{-3}\text{)} = \frac{\text{Mean wt. of gonad in g}}{(\text{Mean shell ht. in mm})^3} \times 10^6$$

Following the introduction into Irish legislation of the Mulroy Bay (North Water and Moross Channel) (Prohibition on Dredging for Escallops) Bye Law No. 625, 1982 to protect broodstock scallops in this site from exploitation and hence conserve the area as a national center for scallop spat collection, farmed scallops from a single year-class and of a size greater than 90 mm shell height were held at 5-m depth on longlines at Deegagh Point in the Broad Water of Mulroy Bay (Fig. 1) and used as a source of adult stock for gonad monitoring.

### Larval Sampling

In 1980 plankton samples for monitoring the occurrence and development of scallop larvae were obtained from a site at Lurgacloghan on the western side of the North Water (Fig. 1) within the site licensed to Deegagh Point Shellfish Ltd. for scallop aquaculture. Samples comprising a single vertical haul were collected using a 63- $\mu$ m mesh plankton net with a 30-cm diameter opening, hauled at a rate of 5 m.min<sup>-1</sup>, from 20 m to the water surface. All planktonic larvae were added to a 1-L, wide-mouthed, plastic jar containing 100 mL of formaldehyde to immediately preserve the living material. In the laboratory, samples were placed in a 1-L beaker and gently stirred. Bivalve larvae, being slightly heavier than most other planktonic organisms, rapidly accumulated in the center of the beaker as a result of the gentle centrifugal force and were transferred to 25 mL Sterilin® storage containers using a 10 mL pipette. Larvae were examined on a Sedgewick Rafter counting cell using  $\times 40$  and  $\times 100$  magnification on an Olympus KH series microscope. Scallop larvae were identified from their morphological features and measured using an eyepiece graticule at  $\times 100$  magnification (Slater 2005b). Identification was possible as a result of expertise gained during hatchery rearing studies with *P. maximus* (L.).

The distribution of scallop larvae in the North Water was investigated by measuring larval abundance at eight sites in the North Water of Mulroy Bay. Samples comprising a single vertical plankton haul from 15 m to the water surface were collected on July 16, 1980 between 10.00 PM to 12.00 PM and repeated on July

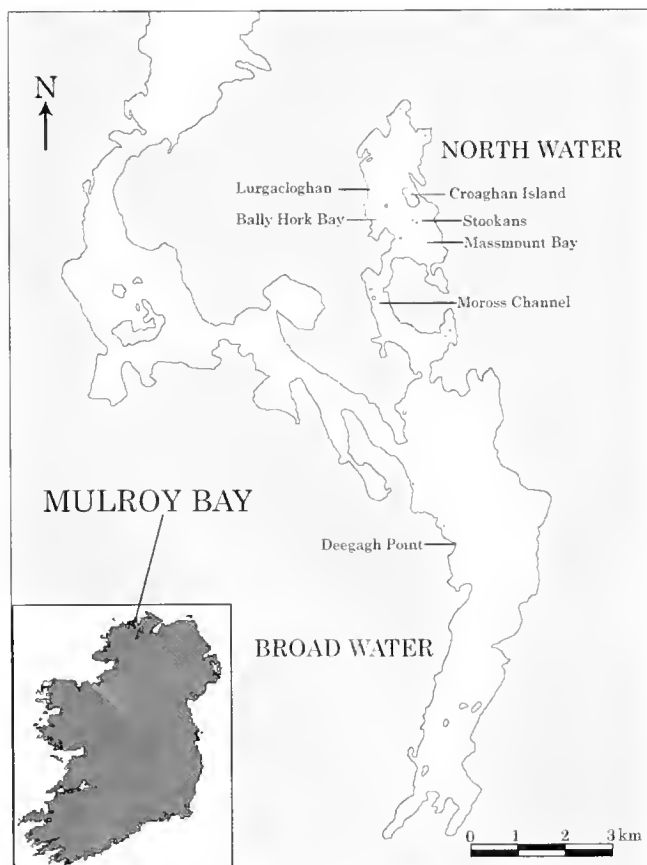


Figure 1. Mulroy Bay on the northwest coast of Ireland.

18, 1980 between 9.00 PM AND 11.00 PM. Weather and sea conditions on both these occasions were ideal for quantitative work of this type.

In 1981 plankton samples for monitoring the occurrence and development of scallop larvae were obtained from the same site at Lurgacloghan on the western side of the North Water using similar techniques to those outlined earlier. A single vertical haul from 20 m to the water surface provided sufficient numbers of scallop larvae for analysis. From 1985 onwards, following use of TBT (tributyl-tin) antifoulants by salmon farms in the Bay, three vertical hauls were collected at this site to provide sufficient scallop larvae for analysis (Minchin et al. 1987).

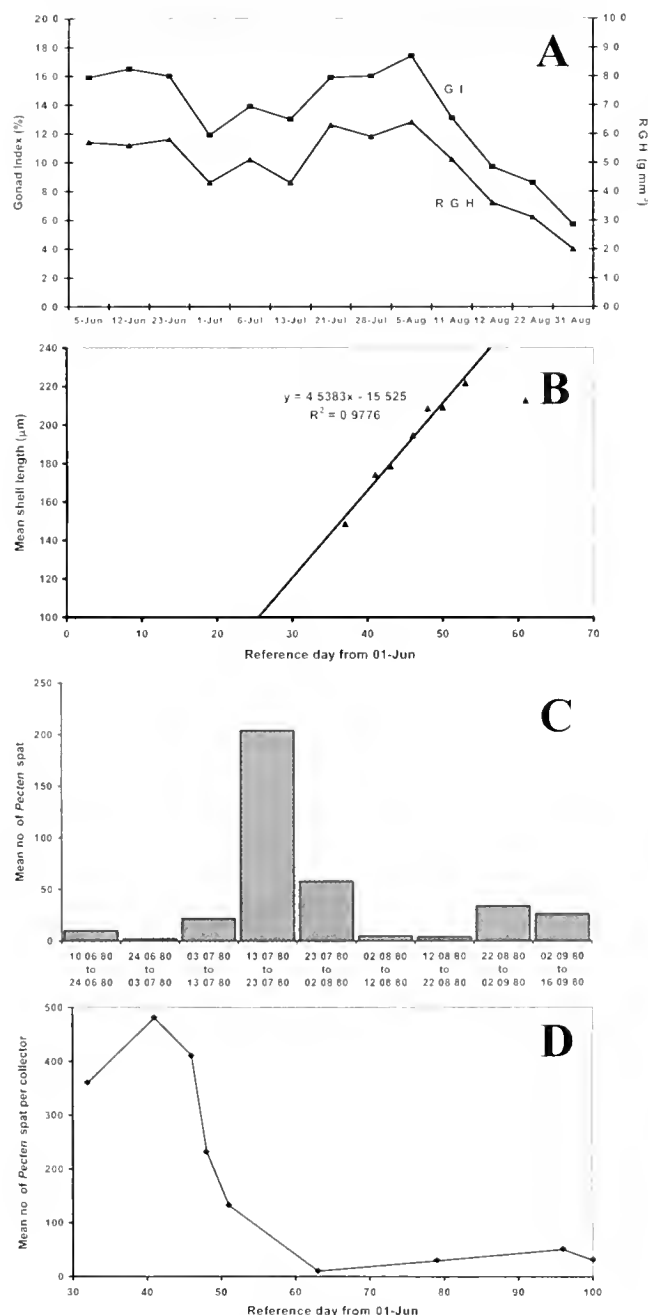


Figure 2. Gonad monitoring (A), larval growth rate (B), spat settlement (C) and scallop spat collection at the site north of Croaghan Island (D) during 1980.

#### Indicator Collectors

Indicator collectors were used to provide data on time and intensity of settlement of scallop spat. These consisted of a piece of 6-mm Netlon® mesh of 0.8 m × 0.3 m dimensions, rolled twice along the long axis into a cylindrical shape and fixed using cable ties.

In 1980 indicator collectors were attached to ropes at 4 m, 8 m and 12 m above the seabed and installed in a 15-m deep site at the north of Croaghan Island (Fig. 1). After 10–11 days immersion, these indicator collectors were exchanged for a new set of collectors for spat settlement. Bivalve spat settling during the 10- to 11-day period were removed from the collectors by immersion in 20% sodium hypochlorite in fresh water for 3 min. The solution was sieved through a 90-μm mesh sieve to collect the recently settled bivalve spat (Davies 1974). Examination of bivalve spat was performed on a Sedgewick Rafter counting cell at ×40 and ×100 magnification. Scallop spat were identified and counted from each indicator collector.

In 1981 trials with indicator collectors were repeated in a 23-m deep site in Bally Hork Bay, the site exhibiting higher spat collection results than the north of Croaghan island site in 1980 (Fig. 1). Collectors were attached at 5 m, 10 m and 15 m beneath the water surface, irrespective of the tidal state, based on the 1980 data on variation in settlement intensity with depth. Because the 1980 results showed a small settlement in early June, the indicator collector trial in 1981 was extended to cover the period May 27 to September 4.

#### Spat Collection

Spat collectors used in 1980 consisted of polyethylene woven-filament mesh bags of 0.45 m × 0.45 m dimensions and mesh size 6 mm × 3 mm. Each mesh bag contained 2 m of monofilament nylon salmon netting provided by the North Western Regional Fisheries Board, following confiscation from illegal fishing activi-

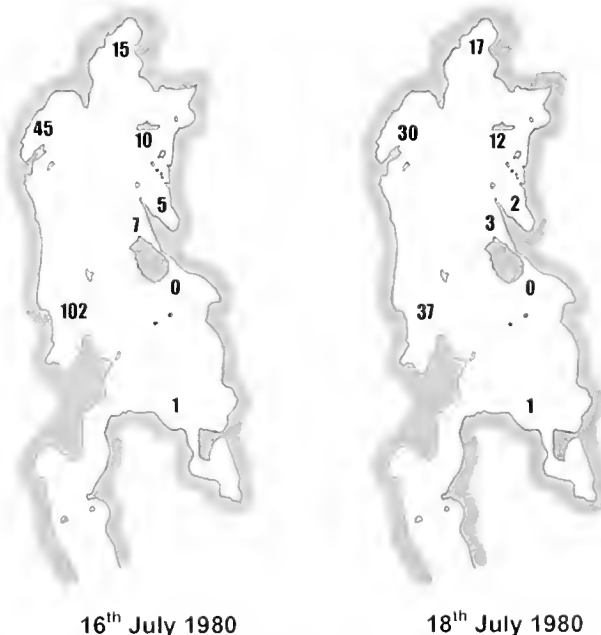


Figure 3. The number of scallop larvae, *P. maximus* in one vertical plankton haul on July 16 and July 18 1980.

TABLE 1.

The number of king scallop spat from collector bags installed between 2–10 m above the seabed at the site north of Croaghan Island between July 2 to September 8, 1980.

Distance Above Seabed (m)	Number of Scallop Spat per Collector Bag									Total Number from Each Depth
	Jul 2	Jul 11	Jul 16	Jul 18	Jul 21	Aug 2	Aug 18	Sept 4	Sept 8	
10	775	920	845	380	200	21	43	41	31	3256
8	440	685	455	270	145	22	38	63	30	2148
6	355	335	440	240	165	6	36	51	41	1669
4	135	320	145	160	90	5	16	66	36	973
2	100	150	170	110	65	2	20	40	20	677
Total number from each date	1805	2410	2055	1160	665	56	153	261	158	
Mean number per collector	361	482	411	232	133	11	31	52	32	

ties. Collector bags were attached to individually anchored 12-mm diameter polypropylene ropes at 2-m intervals from 2–10 m above the seabed to provide information concerning the optimal depth for collection in the 15-m deep site north of Croaghan Island. A 20-cm diameter subsurface float was attached to each rope at 11 m above the seabed to reduce the action of wind and waves on collector bags. A 30-cm diameter marker buoy was attached to the top of the rope to mark the position of the collector line. A total of nine such ropes were positioned in the site from July 2 to September 2, 1980.

Following the larval distribution study, two ropes, each with five collector bags attached as previously mentioned, were positioned on July 17, 1980 in Bally Hork Bay, the site with the highest larval numbers. Because of limited supplies of the polyethylene woven-filament collectors, collector bags at this site comprised 1 m × 0.5 m Netlon® oyster bags of 6 mm × 6 mm mesh size containing 6 m of monofilament mesh salmon netting with the ends of the bags closed at right angles to each other. The spat collection efficiency of this type of collector, which had been successfully used in previous Scottish trials, was compared with the polyethylene woven filament collector in the north of Croaghan island site. Sorting of all collector bags commenced from late October onwards and continued over a two-week period. Collector bags and inner net filler were individually washed into tanks of seawater and live scallop spat from each bag counted individually.

Following the 1980 trials, which demonstrated increased numbers of *Pecten* spat with increased distance from the seabed at two sites in the North Water, collector bags used during 1981 were positioned at 2-m intervals from 2 m to 10 m beneath the water surface. All collector bags used were of the polyethylene woven-filament type and were positioned in Bally Hork Bay, the more productive of the two sites in the North Water and four sites in the Broad Water investigated during 1980.

Spat collection data from 2002 were provided by the pioneering scallop farming company, Deegagh Point Shellfish Ltd., established in 1980 to commercially exploit king scallop spat collection in Mulroy Bay. Collector bags used for commercial spat collection were 0.8 m × 0.3 m dimensions and 5 mm × 3 mm mesh size and filled with 2 m × 1 m of lightweight garden netting (Netlon®) of 6 mm × 6 mm mesh size. All collector bags were suspended from 300 m longlines, submerged 4 m beneath the water surface and attached to the shore at one end in a site at Lurgacloghan (Fig. 1). Spat collectors were installed over a 3-day period at the date of peak spatfall determined using gonad analysis and scallop larval monitoring.

## RESULTS

### 1980 Gonad Monitoring

A large partial spawning was recorded from June 23 to July 1, GI decreasing from 16.0% to 11.9%. Following a rematuration phase over one week, a second smaller partial spawning was recorded from July 6 to 13, with GI decreasing from 13.9% to 13.0%. This smaller second partial spawning was more clearly evident in the RGH results, which accounted for shell size, there being a 3-mm increase in shell length of scallops used for the assessment on July 13, compared with July 6. Following gonad filling during the remainder of July, a larger and more complete spawning commenced in early August and continued throughout that month (Fig. 2A).

The trend in results for gonad index (GI), which relates the gonad weight to the total soft tissue weight, was very similar to that for relative gonad height (RGH), which relates the gonad weight to the size of the adult shell (Fig. 2A). There was a highly significant correlation between GI and RGH ( $F_{1,11} = 300.158$ ,  $P < 0.001$ ).

### 1980 Larval Monitoring

Larvae of the scallop, *Pecten maximus*, were first recorded on July 7 at a mean shell length of 148.5  $\mu\text{m}$  and were present in all samples until July 31. The number of scallop larvae measured in each sample ranged from 3–100 during this period. Larval growth rate was approximately 4.54  $\mu\text{m} \cdot \text{day}^{-1}$  (Fig. 2B). Extrapolation of

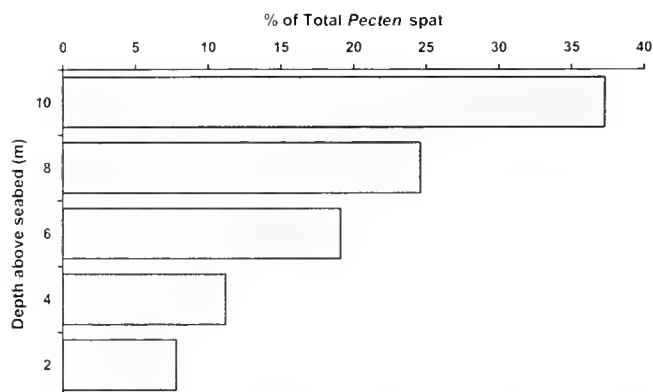


Figure 4. Variation in king scallop spat collection with depth above the seabed at the site north of Croaghan Island during 1980.



the larval growth trendline to a shell length of 100  $\mu\text{m}$ , a size usually achieved in the hatchery at three days old, suggested that these larvae originated from a spawning on June 22, corresponding with the first partial spawning recorded by gonad monitoring between June 23 and July 1. The final result on July 31 of 212.7  $\mu\text{m}$ , based on only three larvae, showed a decline from the previous result on July 23 of 221.7  $\mu\text{m}$ . This decline was attributed to the settlement of larger larvae from the plankton at the same time as the few remaining planktonic larvae continued to grow.

Quantitative assessment of the distribution of scallop larvae on July 16 and July 18 showed greater abundance of scallop larvae in Bally Hork Bay (Fig. 3).

Scallop larvae on the two sampling dates had an average shell

TABLE 2.

Abundance and mean shell length data of two hatches of scallop larvae *P. maximus* in the North Water of Mulroy Bay during 1981.

Date	Ref. Day From Jun 01	Larval Batch 1		Larval Batch 2	
		n	Mean Shell Length ( $\mu\text{m}$ )	n	Mean Shell Length ( $\mu\text{m}$ )
01.06.81	1				
20.06.81	20	8	146	—	—
22.06.81	22	19	155	—	—
24.06.81	24	22	166	—	—
26.06.81	26	8	175	—	—
28.06.81	28	59	178	—	—
28.06.81	30	9	190	—	—
02.07.81	32	16	200	—	—
03.07.81	33	16	202	—	—
07.07.81	37	12	206	88	154
08.07.81	38	8	210	92	156
10.07.81	40	6	217	144	168
13.07.81	43	—	—	100	186
15.07.81	45	—	—	144	198
18.07.81	48	—	—	25	200
20.07.81	50	—	—	70	201
24.07.81	54	—	—	28	196
27.07.81	57	—	—	79	202
30.07.81	60	—	—	15	197

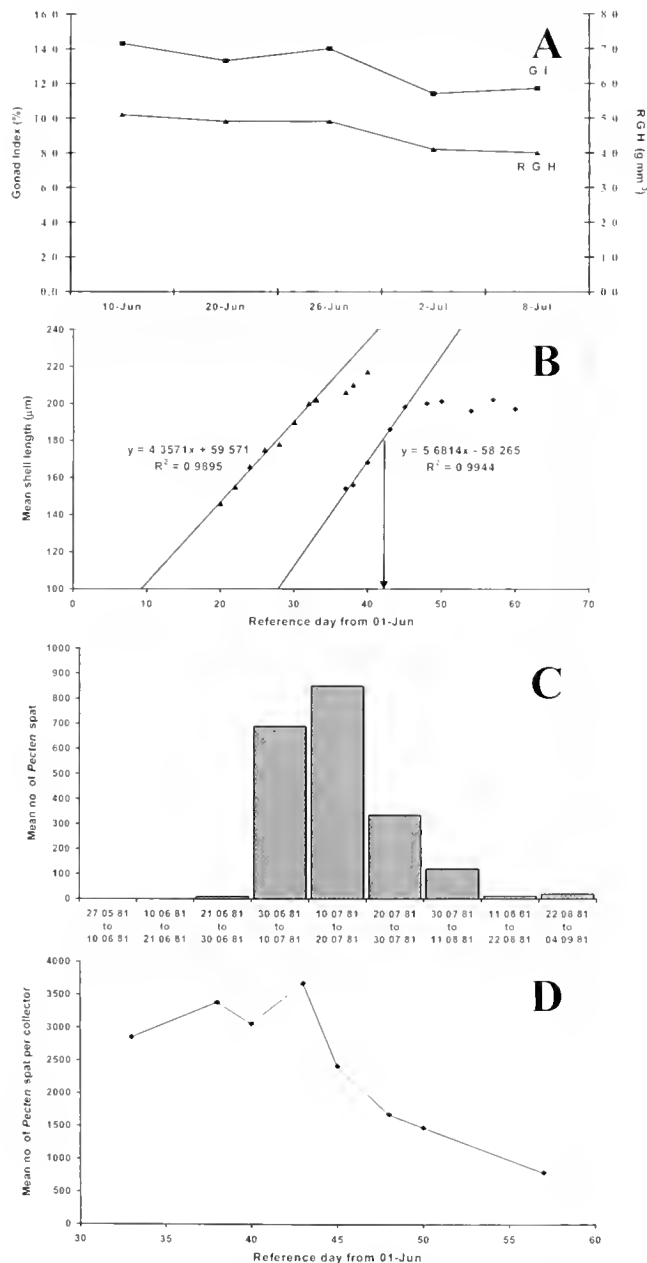


Figure 5. Gonad monitoring (A), larval growth rate (B), spat settlement (C) and scallop spat collection at the site in Bally Hork Bay (D) during 1981.

length of 194.5  $\mu\text{m}$  and 208.5  $\mu\text{m}$  respectively (i.e., near settlement size).

#### 1980 Indicator Collectors

Low numbers of scallop spat settled on the first set of indicator collectors immersed on June 10 and lifted on June 24 in the site north of Croaghan Island, showing that some spawning activity had occurred prior to June 10. Peak settlement intensity occurred between July 13–23 with an average of 204 spat per collector (Fig. 2C). Settlement intensity varied with distance above the seabed in this 15-m deep site with the indicator collectors situated 8 m above the seabed (i.e., in midwater exhibiting the highest settlement intensity).

#### 1980 Spat Collectors

Results of the spat collection trial at the site north of Croaghan Island, a site exhibiting only 7 and 3 scallop larvae on July 16 and July 18 respectively are provided in Table 1. Peak scallop spat collection occurred from July 11–16 with an average of 450 *Pecten* spat per collector (Fig. 2D). Given a 20-day larval life for this species, a typical value from hatchery production of this species, peak spat collection corresponded to the large partial spawning recorded from June 23 to July 1. A much smaller peak in spat collection on September 4 yielding approximately 50 *Pecten* spat per collector corresponded to the more complete August spawning. Spat collectors installed in Bally Hork Bay, the site with the highest larval counts on July 16 and July 18, yielded an average of 2,100 *Pecten* spat per collector, although these results are not comparable because different collectors were used in the two sites. In both sites increased numbers of *Pecten* spat were obtained with increased distance of the collector bag above the seabed (Fig. 4).

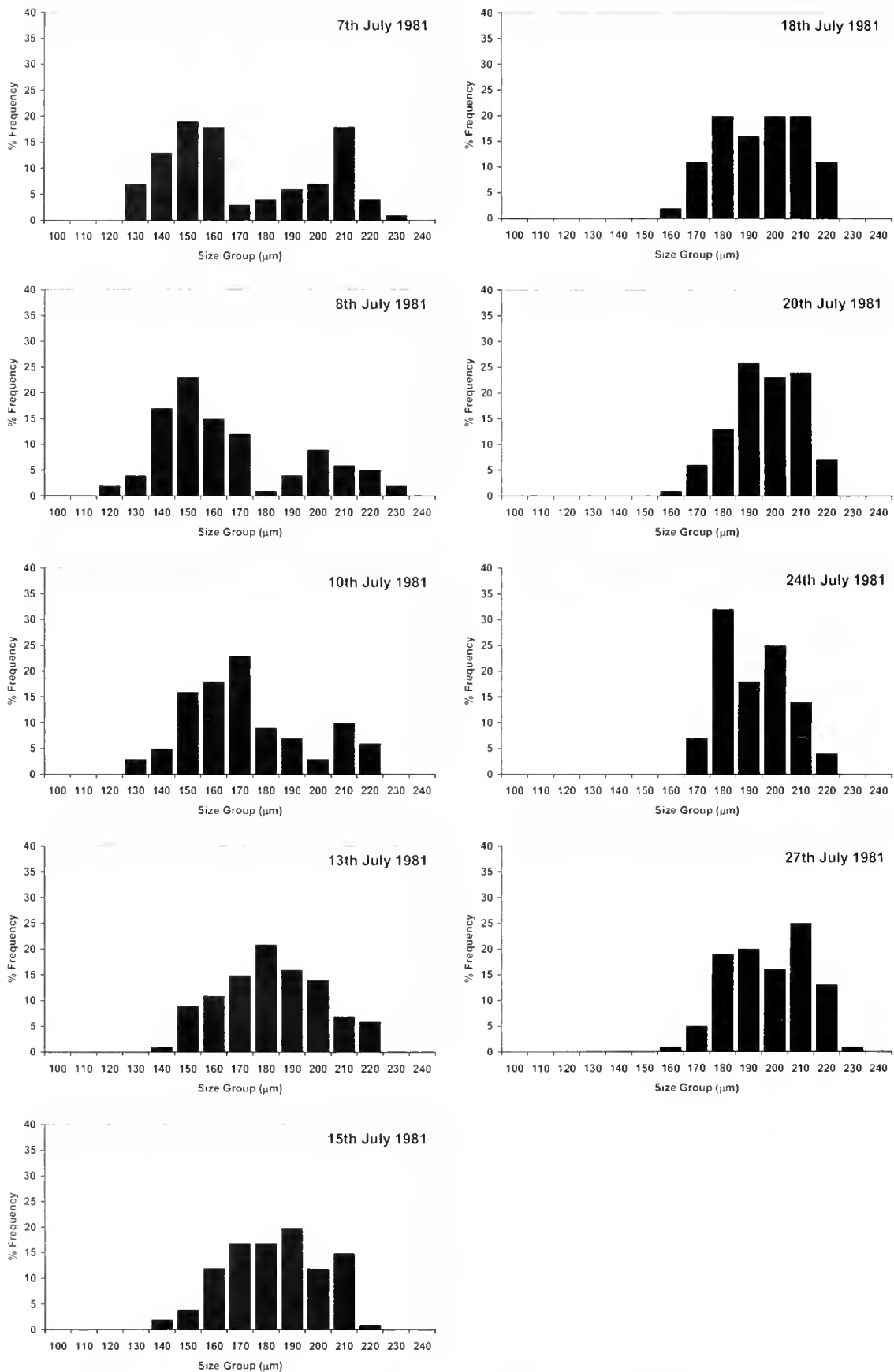


Figure 6. Size distribution analysis of *P. maximus* larval shell length from July 7 to July 27, 1981.

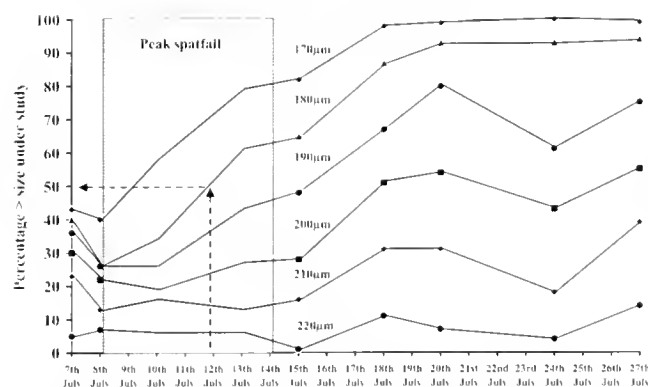


Figure 7. Variation in the percentage of *P. maximus* scallop larvae  $\geq 170$   $\mu\text{m}$ , 180  $\mu\text{m}$ , 190  $\mu\text{m}$ , 200  $\mu\text{m}$ , 210  $\mu\text{m}$  and 220  $\mu\text{m}$  in Bally Hork Bay during 1981.

#### 1981 Gonad Monitoring

A small partial spawning occurred between June 10–20, with GI decreasing from 14.3% to 13.3%, followed by a short rematuration phase and a second larger partial spawning from 26 June to July 2 with GI decreasing from 14.0% to 11.4% (Fig. 5A). Similar to 1980 there was a highly significant correlation between GI and RGH in 1981 ( $F_{1,5} = 1083.71$ ,  $P < 0.001$ ).

#### 1981 Larval Monitoring

Larvae of the scallop, *P. maximus*, were first recorded on June 20 at a mean shell length of 146  $\mu\text{m}$  (Table 2). On the July 7 size distribution analysis of larvae showed the presence of two larval batches (Fig. 6). The larval batch comprising larger-sized larvae was not recorded after July 13. Between July 13 to July 30 size distribution analysis showed the presence of only one larval batch.

Scallop larval growth rates were approximately  $4.36 \mu\text{m} \cdot \text{day}^{-1}$  for the first larval batch and  $5.68 \mu\text{m} \cdot \text{day}^{-1}$  for the second larval batch (Fig. 5B). Extrapolation of the larval growth trendlines to a shell length of 100  $\mu\text{m}$ , a size usually achieved in the hatchery at three days old, suggested that the first larval batch originated from a spawning on June 6, a time prior to the commencement of scallop gonad monitoring and the second larval batch originated from a spawning on June 25 corresponding with the spawning recorded using GI between June 26 to July 2. Above 200  $\mu\text{m}$  both larval batches exhibited deviations from the straight line best-fit attributed to the settlement of larger larvae from the plankton at the same time as remaining planktonic larvae continued to grow.

Larval shell length data from the more abundant second batch of larvae (Table 2) were analyzed in terms of the percentage of larvae greater than or equal to 170  $\mu\text{m}$ , 180  $\mu\text{m}$ , 190  $\mu\text{m}$ , 200  $\mu\text{m}$ , 210  $\mu\text{m}$  and 220  $\mu\text{m}$  on each sampling occasion between July 7 and 27 (Fig. 7). The objective of this analysis was the development of a predictive model similar to that used in Japan in which a critical size and a percentage greater than or equal to that size are used to identify the date of the peak spat collection.

#### 1981 Indicator Collectors

Data from indicator collectors in Bally Hork Bay demonstrated that scallop spat settlement during May and June was negligible. Heavy settlement intensity occurred on the two sets of indicator collectors used during the period June 30 to July 20, with a mean settlement intensity of 688 and 849 spat per indicator collector respectively (Fig. 5C). Settlement intensity varied with distance from the water surface in this 23-m deep site with the indicator collectors situated 10 m below the water surface exhibiting the highest settlement intensity.

#### 1981 Spat Collectors

Spat collection in Bally Hork Bay peaked from July 8–14, slightly earlier than in 1980 (Table 3, Fig. 5D). Collector bags immersed during this period yielded over 3,000 spat per collector. Spatfall corresponded with the spawning period identified by GI from June 26 to July 2 and the spawning date on June 25 estimated by extrapolation of the larval trendline from the second larval batch monitored over the period July 7–30. Peak scallop spat collection at the Bally Hork Bay site in 1981 occurred 4 m beneath the water surface (Fig. 8).

#### Summary of the 1980 and 1981 Experimental Programs

The 1980 experimental program demonstrated that a spawning from June 23 to July 1 resulted in larvae that were first identified on July 7 at a mean shell length of approximately 150  $\mu\text{m}$ . Larval growth rate was  $4.54 \mu\text{m} \cdot \text{day}^{-1}$ . Spat collection intensity peaked from July 11–16, a time at which the mean shell length of larvae was between 174  $\mu\text{m}$  to 194.5  $\mu\text{m}$ . Increased spat collection intensity occurred in Bally Hork Bay, a site in which the highest larval concentration was recorded, compared with the north of Croagh Island site. In both sites increased spat collection occurred with increased distance from the seabed.

The 1981 experimental program reported two spawnings from June 10 to 20 and June 26 to July 2 resulting in larvae from each

TABLE 3.

The number of king scallop spat from collector bags installed between 2–10 m below the water surface at the site in Bally Hork Bay between July 3–27, 1981. (N/A = Collector bags missing at the time of retrieval.)

Distance Below Water Surface (m)	Number of Scallop Spat per Collector Bag								Total Number from Each Depth
	Jul 3	Jul 8	Jul 10	Jul 13	Jul 15	Jul 18	Jul 20	Jul 27	
2	3060	4060	3260	3865	N/A	N/A	1355	620	16220
4	3010	3640	3320	5070	N/A	N/A	2020	790	17850
6	2905	3530	3320	3900	2670	N/A	1730	930	18985
8	2540	3275	3230	3080	1970	N/A	1220	860	16175
10	2750	2430	2135	2460	1555	1080	1040	765	14215
Total number from each date	14265	16935	15265	18375	6195	1080	7365	3965	
Mean number per collector	2853	3387	3053	3675	2065	1080	1473	793	

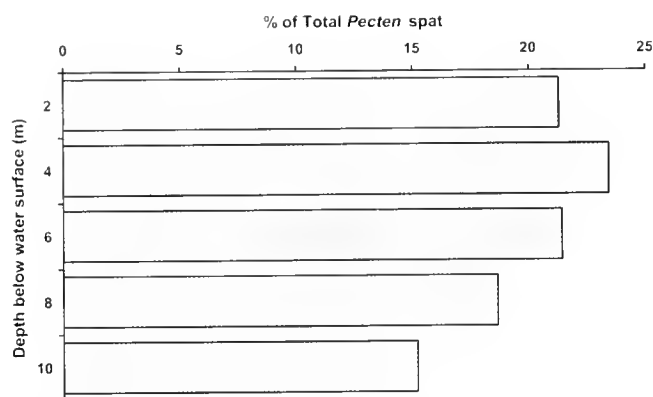


Figure 8. Variation in king scallop spat collection with depth from the water surface at the Bally Hork Bay site during 1981. (Data from the July 15 and 18 excluded because of missing collectors).

that were first identified on June 20 and July 7 respectively at a mean shell length of approximately 150  $\mu\text{m}$ . Larval growth rate for the two larval batches were 4.36  $\mu\text{m}\cdot\text{day}^{-1}$  and 5.68  $\mu\text{m}\cdot\text{day}^{-1}$ . Spat collection intensity in Bally Hork Bay peaked from July 8 to 14, a time at which the mean shell length of larvae was between 156  $\mu\text{m}$  to 194.5  $\mu\text{m}$ . Scallop spat collection peaked 4 m beneath the water surface.

Both experimental programs demonstrated that peak spat collection could be predicted using a combination of gonad monitoring and larval development. Gonad monitoring demonstrated that a spawning had occurred, though this did not necessarily infer that significant numbers of larvae has been produced or that spat collection would occur. Larval development was more useful as a means of spatfall prediction, because it focused on the developmental stage immediately prior to settlement. Based on the data from the two years of experimental investigation a hypothesis was proposed that the date of peak spat collection occurred when the mean shell length of larvae was 180  $\mu\text{m}$  (Fig. 5B) and 50% of the larval cohort exceeded 180  $\mu\text{m}$  (Fig. 7). Using these two models the dates for peak spat collection in 1981 would have been predicted as the July 11–12 by the mean shell length model and July 11–12 by the percentage greater than 180- $\mu\text{m}$  model. The installation of collector bags on these dates would have resulted in excellent yields of scallop spat in 1981.

#### Commercial application of the technique in 2002

Since its development in the early 80s these techniques for prediction of the date of the scallop spatfall have been used commercially in the North Water of Mulroy Bay. Data from the 2002 commercial collection of spat have been provided for illustrative purposes. Monitoring of GI and RGH of scallops indicated that a partial spawning occurred from July 4 to July 11, 2002 with GI decreasing from 15.6% to 14.0% (Fig. 9A).

Low numbers of larvae of the scallop, *Pecten maximus*, were recorded on June 24, the first day of sampling at a mean shell length of 133.6  $\mu\text{m}$  (Table 4). The growth rate of these larvae was approximately 4.19  $\mu\text{m}\cdot\text{day}^{-1}$  (Fig. 9B). Extrapolation of the larval growth trendline suggested that this larval batch originated from a spawning on June 13, a time prior to the commencement of gonad monitoring. Size distribution analysis of larvae collected on July 9 showed the presence of an abundant larval batch of shell length 120  $\mu\text{m}$  to 140  $\mu\text{m}$  and the presence of at least one less abundant cohort corresponding to an earlier spawning(s). Micro-

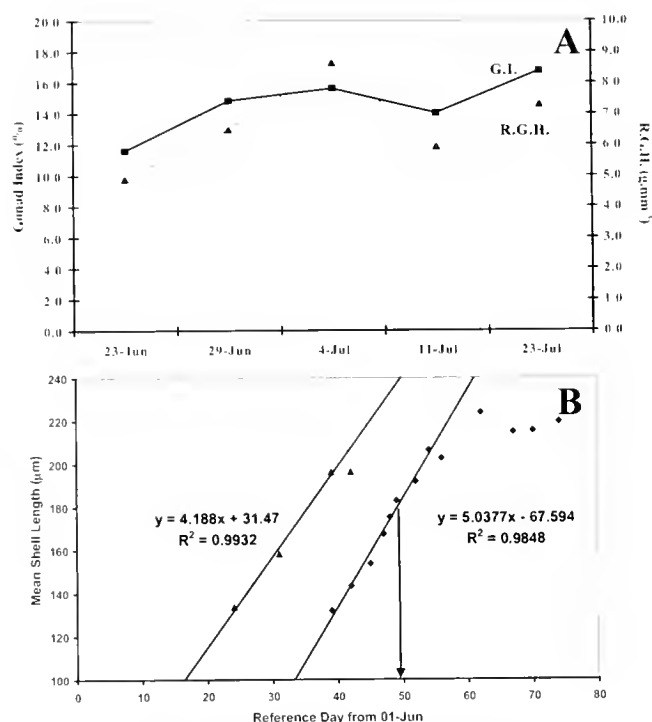


Figure 9. Gonad monitoring (A) and larval growth rate at Lurgaloghan in the North Water of Mulroy Bay (B) during 2002.

analysis of the size distribution data of samples from July 15 to July 22 could be used to suggest that these scallop larvae represented one, two, three or even four larval cohorts with overlapping size distributions. However, because each larval size distribution in this period represented from 28–100 larval measurements, a number considered too low for microanalysis of data, this was not performed in this study (Fig. 10). Instead larvae from July 9 and July 12, samples have been considered as comprising two larval batches and larvae from July 15 onwards as one larval

TABLE 4.

Abundance and mean shell length data of two batches of scallop larvae *P. maximus* in the North Water of Mulroy Bay during 2002.

Date	Ref. Day from Jun 01	Larval Batch 1		Larval Batch 2	
		n	Mean Shell Length ( $\mu\text{m}$ )	n	Mean Shell Length ( $\mu\text{m}$ )
24.06.02	24	29	133.6	—	—
01.07.02	31	7	158.3	—	—
09.07.02	39	5	196.2	95	131.9
12.07.02	42	5	196.4	95	143.5
15.07.02	45	—	—	28	153.8
17.07.02	47	—	—	100	167.4
18.07.02	48	—	—	84	175.4
19.07.02	49	—	—	63	183.1
22.07.02	52	—	—	100	191.9
24.07.02	54	—	—	66	206.5
26.07.02	56	—	—	40	202.6
01.08.02	62	—	—	13	223.8
06.08.02	67	—	—	9	214.9
09.08.02	70	—	—	13	215.5
13.08.02	74	—	—	7	219.6

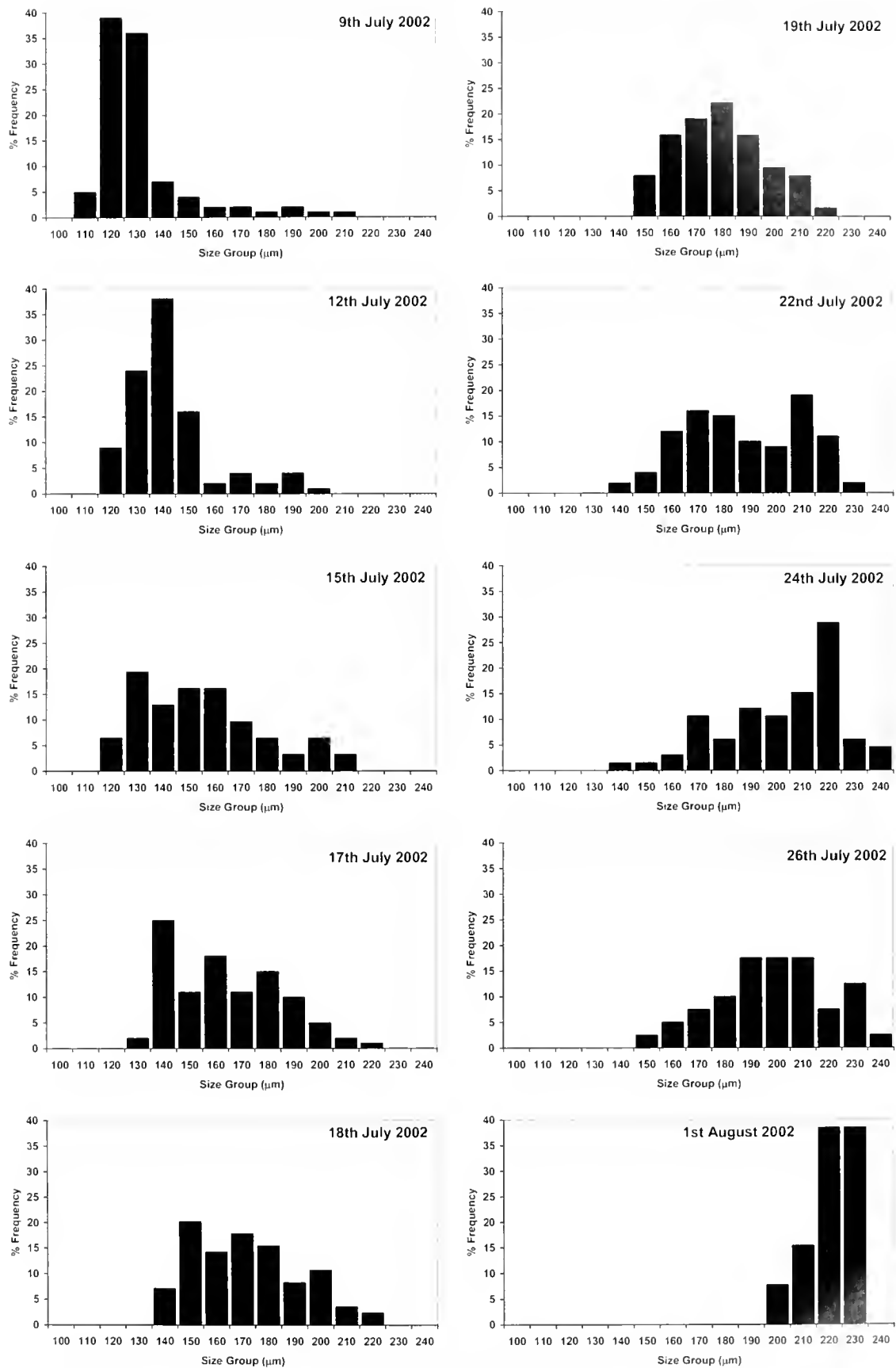


Figure 10. Size distribution analysis of *P. maximus* larval shell length between July 9 and August 1, 2002.

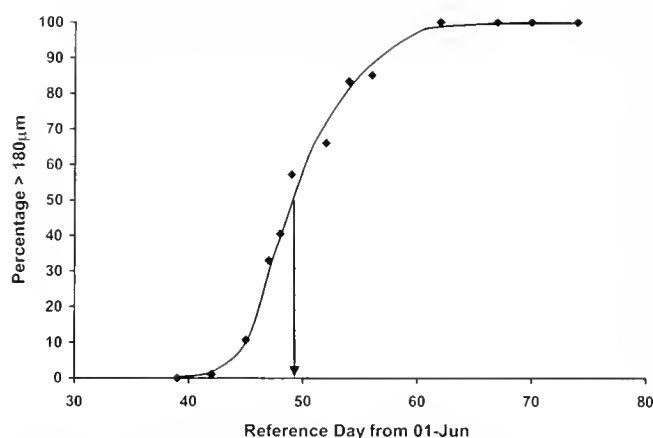


Figure 11. The percentage of *P. maximus* larvae >180 µm from the second larval batch during 2002.

batch. The growth rate of larvae in the second, more abundant, larval batch was approximately  $5.04 \mu\text{m}\cdot\text{day}^{-1}$  (Fig. 9B). Extrapolation of the larval growth trendline suggested that the second larval batch originated from a spawning on June 30, somewhat earlier than the July 4–11 spawning period identified using GI monitoring. Growth rate of the second larval batch, as in 1980 and 1981, decreased above 200 µm and was attributed to the settlement of larger larvae at the same time as the remaining planktonic larvae continued to grow. Using the prediction model developed in 1981, date of peak spat collection in 2002 was forecast as July 19, using both the mean shell length model (Fig. 9B) and the 50% greater than 180 µm model (Fig. 11).

Commercial scallop spat collection yields in excess of 3,000 per collector bag were obtained from collector bags installed on July 19, 2002 and sorted in October 2002.

## DISCUSSION

Determination of the spawning time of the scallop, *P. maximus* and thus the time at which larvae should be present in the water can be elucidated using either GI or RGH calculated from pooled tissue weights and mean shell size. The benefit of using more than one measure of reproductive condition was evident from the results of the second spawning in 1980; the small difference in GI results being more clearly evident in the RGH results, which accounted for the increased shell length between the samples. Although individual tissue weights and shell sizes would have provided a measure of the dispersion in reproductive condition within the sample, analytical equipment required for individual measurement was not available in 1980 and 1981. Frequent gonad monitoring at regular weekly intervals was used to ensure the provision of meaningful data. Partial spawnings during the summer, which can be all important in terms of scallop spat production, may be of short duration and be followed by rapid rematuration to pre-spawning levels (Slater 2005a). Sampling intervals greater than weekly could fail to record such partial spawnings. Even with a weekly sampling interval, gonad monitoring alone is not suitable as a tool for spatfall prediction. For example, in 1980 three spawnings were recorded over the duration of the sampling program, however significant numbers of scallop larvae were only recorded from the larger of the two partial spawnings occurring between late June to early July. These results demonstrate one inherent weakness of using gonad monitoring alone, namely that based on gonad results

it would be reasonable to assume that three spawnings should have resulted in three larval batches and hence three spatfalls, with the larger more complete spawning in late August possibly providing the largest spat collection.

Scallop larval growth rates under natural conditions of  $4.54 \mu\text{m}\cdot\text{day}^{-1}$  in 1980,  $4.36 \mu\text{m}\cdot\text{day}^{-1}$  and  $5.68 \mu\text{m}\cdot\text{day}^{-1}$  for the two batches in 1981, and  $4.19 \mu\text{m}\cdot\text{day}^{-1}$  and  $5.04 \mu\text{m}\cdot\text{day}^{-1}$  in 2002 were comparable with figures reported from hatcheries (Comely 1972, Gruffydd & Beaumont 1972, Le Penneec 1974, 1978). Back extrapolation of the larval growth rate trendline can be used to estimate the spawning date. Comparison of spawning dates provided by gonad monitoring with those provided by back extrapolation of the larval growth rate revealed a second weakness of the former, namely that GI/RGH identified a "spawning period" within which spawning was assumed to have occurred, whereas back extrapolation of the larval growth rate provided a "spawning date." The occurrence of a "spawning date" prior to the commencement of the "spawning period" (Table 5) may occur as an artifact of the sampling frequency used in gonad monitoring, spawning having already commenced before the high GI result at the commencement of the "spawning period" but not having been recorded because of the sampling frequency.

In summary, gonad monitoring can at best be used to provide an indication of the time period when larvae may be present, whereas larval monitoring can be used to provide confirmation that spawning has occurred and that scallop larvae have been produced and are developing normally.

To obtain an indication of the spatial distribution of scallop larvae, numbers of scallop larvae in a single vertical haul from 15 m to the water surface at eight sites in the North Water were determined. Other studies have reported that for quantitative work, plankton samples collected with a pump rather than plankton net provide a better estimate of abundance, however such equipment was not available for this study. Although significant variation in larval numbers between hauls have been reported in the literature, a single haul in the late evening was used in this investigation to allow rapid examination of all samples and the installation of collectors the following day in the site showing highest larval abundance. Quantitative analysis of the numbers of scallop larvae in a single vertical haul in eight sites demonstrated similar distribution patterns in the North Water on two dates in July 1980 (Fig. 3). The higher spat collection intensity in Bally Hork Bay com-

TABLE 5.

Summary of scallop spawning "dates" using larval growth extrapolation and spawning "periods" using gonad index in 1980, 1981 and 2002.

	Spawning "Date" from Larval Growth Extrapolation	Spawning "Period" from Gonad Index
1980 larvae	June 22	June 23–July 1
1981 larvae (batch 1)	June 6	Before sampling commenced
1981 larvae (batch 2)	June 25	June 26–July 2
2002 larvae (batch 1)	June 13	Before sampling commenced
2002 larvae (batch 2)	June 30	July 4–July 11

pared with north of Croaghan Island, the former site having the highest larval abundance on both of the quantitative sampling dates, suggested that quantitative larval assessment using a single vertical haul with a plankton net could be used to provide an indication of larval distribution in this sheltered location and selection of more productive areas for scallop spat collection. A similar study into the distribution of larvae of *Patinopecten yessoensis* in Mutsu Bay, Japan reported that after initial widespread distribution in all regions, larvae accumulated in the eastern part of the Bay where spat settlement was highest (Ito et al. 1975). Changes in larval spatial distribution as larvae increased in size were attributed to regional water currents (Ventilla 1980).

The series of indicator collectors immersed for 10-day periods throughout the June to August period in 1980 exhibited three peaks in settlement, a small peak occurring in early June, the main settlement peak occurring in mid July and a small peak occurring in late August. In 1981, the main settlement peak again occurred in mid July, however the early June peak appeared to have moved to late June and the late August peak was of a smaller size. Experimental results for these two years demonstrated the dominant mid July peak settlement compared with other peaks in settlement activity. The importance of installing collectors at the time of maximum settlement is clearly exhibited in the 1980 results with a 9-fold increase at peak settlement time compared with the previous 10-day period and a 4-fold increase compared with the 10-day period after peak settlement. Similar 5-fold changes in spat settlement intensity within a 10-day period of the peak settlement have been reported with Japanese scallop, *P. yessoensis* in Mutsu Bay (Kanno 1970, Ito 1977). Such wide variation in spat settlement intensity over a short time period either side of the peak settlement raises doubts about the validity of results of much of the published work undertaken with natural spat collection where the poor settlements reported in some years could be attributed to the installation of collectors in the wrong time period (Mason 1969, Minchin 1981, 1983, Brand et al. 1980, Cashmore et al. 1998, McDonough 1998, Maguire & Burnell 1999).

Indicator collectors have no role in prediction of the time of the scallop spatfall because their results are only available postsettlement. Nevertheless, as a tool for optimizing site selection, for investigating peak settlement depths or providing an early indication of settlement intensity as a means of estimating equipment requirements for intermediate culture they can be of significant value.

Spat collection results from collector bags in 1980 and 1981 reflected the pattern of results obtained with indicator collectors. A sharper peak in spat collection intensity occurred in 1980 compared with 1981 and is attributed to movement of the early June settlement peak to late June during the 1981 season. The 1980 and 1981 results from Bally Hork Bay and the commercial result from 2002 demonstrated that the North Water of Mulroy Bay in County Donegal has significant development potential as a scallop spat collection center and is capable of exceeding the 1,000 spat per collector bag level identified in Japan as the standard for good and poor collection years (Taguchi & Walford 1976).

The experimental results obtained in 1980 and 1981 provided the foundation for a scallop spatfall prediction technique for use in Mulroy Bay. Since its development, the technique has been applied commercially resulting in the provision of millions of scallop spat for on-growing trials by the fledgling scallop culture industry in Ireland. The prediction technique involved the installation of spat collectors when the mean shell length of scallop larvae was

180  $\mu\text{m}$  and 50% of the larval batch exceeded this size. By comparison, in Mutsu Bay with the Japanese scallop, *P. yessoensis*, the time recommended for the installation of collectors to correspond with peak collection is when 50% of umbonate larvae exceed 200  $\mu\text{m}$  in size (Ventilla 1980). In previous studies in Scotland with *P. maximus*, a mean larval shell length of 200  $\mu\text{m}$  was shown to correspond with the peak spat collection of this scallop (Slater 1980). These differences in the critical size, even with the same species, are attributed to the difference in settlement size of the larvae, settlement in Mulroy Bay occurring at approximately 220  $\mu\text{m}$  compared with 245–250  $\mu\text{m}$  in Scotland and 280–300  $\mu\text{m}$  with *P. yessoensis* in Japan.

Other techniques for prediction of the scallop spatfall in Japan have been developed. Yamamoto (1964) reported an association between the spatfall time and cumulative water temperatures over 4°C, which allowed prediction of the scallop spawning period and thus the spat settlement period. Ito et al. (1975) reported a relationship between the date of the scallop spatfall and the blooming of cherry blossom trees in Gappo Park, Aomori City. Because the Meteorological Department issued the blooming forecast one month in advance for the different regions of Japan, spat settlement dates could also be predicted. Although reported in the literature, these methods are capable of providing only a general guide to the settlement date (Ventilla 1980).

Peak collection of scallop spat in Bally Hork Bay, a site with a water depth of 23 m, occurred 4 m beneath the water surface in 1981. By contrast, most other studies have reported peak scallop spat collection with this species nearer the seabed (Buestel et al. 1976; Ventilla 1977a; 1977b; Slater 1978; 1979; 1980; Brand et al. 1980; Thouzeau 1991) or in midwater (Burnell et al. 1991, Maguire & Burnell 1999). Comparison of the peak depth of settlement on indicator collectors and the peak depth of collection on collector bags did not provide any clear explanation for the difference in depth profile between Mulroy Bay and the other sites reported in the literature. One factor that may contribute to the differences between sites may be the calmer less turbulent waters in Mulroy Bay, resulting in a reduced loss of spat nearer the water surface by comparison with the more open water sites reported elsewhere (Pearce et al. 1998). Recently the intensity of water column stratification, which might be more intense in calmer waters, was shown to influence the depth distribution of scallop larval settlement (Pearce et al. 2004).

On a note of caution regarding the commercial application of data of this nature, difficulties in the management of this unique scallop resource resulted when some of the results from the 1980 and 1981 investigations were made available. For example, local awareness of the fact that scallop larval concentrations were highest in Bally Hork Bay resulted in an over-focus on this area, with every scallop farming entrepreneur demanding access to this site. This resulted in the installation of longlines and collector bags across each other, threatening behavior between potential farmers, malicious damage to equipment and unfortunately compensation claims for damages through the courts. Good publicity directed at the potential of scallop farming combined with adverse media coverage focused on the court actions attracted the attention of commercial divers and resulted in the illegal removal of much of the scallop broodstock, despite the fact that diver removal of shellfish is prohibited in Ireland. The diminished broodstock population resulting from diver removal coupled with the effects of poor recruitment caused by the use of TBT antifoulants on salmon cage nets during the mid 1980s (Minchin et al. 1987) resulted in reduced

numbers of scallop larvae and necessitated the collection of three vertical plankton hauls to ensure sufficient larval numbers in plankton samples for application of the prediction technique. Despite these difficulties the spatfall prediction techniques developed have been demonstrated over the last 20 years to be an effective technique for prediction of the date of peak scallop spat collection.

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## HETEROSIS BETWEEN TWO STOCKS OF THE BAY SCALLOP, *ARGOPECTEN IRRADIANS* LAMARCK (1819)

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**ABSTRACT** Heterosis between two stocks (A and B) of the bay scallop *Argopecten irradians irradians* (Lamarck, 1819) was studied by comparing performance in hatching success, larval survival, larval, spat and adult growth of reciprocal hybrid crosses (AB and BA) and that of two parental groups (AA and BB). Stock A is derived from the initial 1982 introduction from Connecticut, USA and expected to be highly inbred because of the small founder population (26 individuals) and the long breeding history (20 + generations). Stock B is the fourth generation from a 1999 introduction from Virginia and Massachusetts, USA, which is expected to have higher heterozygosity caused by a larger founder population (200 wild and 206 first generation cultured individuals) and a short breeding history. Both hybrid crosses demonstrated positive heterosis for all traits studied, although the magnitude of heterosis varied among traits and life history stages. Midparent heterosis was 7.9% for hatching success, 10.8% and 3.0% for larval survival and growth and 8.4% and 10.9% for juvenile and adult growth. Single-parent heterosis also varied among traits. For fitness related traits such as hatching success and larval survival, single-parent heterosis was positive for both parental stocks. Single-parent heterosis for growth, a morphological trait, was positive in stock A and negative in stock B.

**KEY WORDS:** heterosis, scallop, *Argopecten irradians irradians*, stocks, pair-crosses

### INTRODUCTION

Observations of heterosis or hybrid vigor (Shull 1914) are centuries old. Shull (1952) defined the term heterosis as "the interpretation of increased vigor, size, fruitfulness, speed of development, resistance to disease and to insect pests, or to climatic rigors of any kind, manifested by crossbred organisms as compared with corresponding inbreds, as the specific results of unlikeness in the constitutions of the uniting parental gametes." This definition, however, is often interpreted as not implying a genetic basis for heterosis, because the definition basically describes the phenotype that results from crossing two different inbred lines (Lamkey & Edwards 1998). Therefore, the definition of heterosis or hybrid vigor as the difference between the F<sub>1</sub>'s and the mean of the two parents (Falconer 1981) is commonly used in the literature, and this heterosis is often called midparent heterosis.

Despite its tremendous success in plant and animal breeding, the genetic basis of heterosis remains uncertain (Griffing 1990, Luo et al. 2001). Three hypotheses of nonadditive gene action may explain heterosis: overdominance (Shull 1908, East 1936), dominance (Bruce 1910) and epistasis (Stubber et al. 1973, Wright 1977). In contrast with overdominance, neither dominance nor epistasis requires heterozygote superiority at any locus (Hedgecock et al. 1996). In spite of the tremendous success of utilizing heterosis in crop improvement (Crow 1998), its exploitation in marine bivalves has been limited and more recent. Heterosis in bivalves was first suggested by Singh and Zouros (1978) based on a positive correlation between allozyme heterozygosity and fitness-related traits in individuals from natural populations. Mallet and Haley (1983) first demonstrated the presence of heterosis in marine bivalves using experimental crosses between different populations of the American oyster *Crassostrea virginica*. Hedgecock et al. (1995) measured heterosis for quantitative traits using crosses among inbred lines of Pacific oyster *Crassostrea gigas*. In recent year, quantitative studies of heterosis using experimental

crosses have been conducted in several marine bivalves (Hedgecock et al. 1996, Cruz & Ibarra 1997, Cruz et al. 1998, Beaumont et al. 2004).

The bay scallop *Argopecten irradians*, a marine bivalve of considerable economic importance, is a functional hermaphrodite that simultaneously spawns eggs and sperm for external fertilization. Like other functional hermaphrodites, self-fertilization is common and can result in rapid inbreeding (Stiles & Choromanski 1995, Zhang et al. 2003). In 1982, the northern subspecies, *A. i. irradians*, was first introduced into China successfully from the United States and developed into one of the most important mariculture industries in China (Zhang et al. 1986). By early 1990, the annual production of bay scallops in China had reached about 200,000 tons (Guo et al. 1999). For many years, however, bay scallop production in China was from the 26 founders introduced in 1982 and as a result, a greater proportion of mtDNA variation was lost in the intervening generations of hatchery breeding (Blake et al. 1997). Additional scallops (406 including 200 of wild and 206 from the first generation of a captivity population) were brought from the United States to China in December 1998 and February 1999 (Gu Z., personal communication), which currently are the main culture stock in China.

In China, bay scallop aquaculture has matured to include a series of well-defined phases such as broodstock conditioning, larval culture, nursery and grow-out. Broodstock conditioning and larval culture are conducted in hatcheries under strict control. Nursery and growth use bags and lantern nets respectively, and adults are harvested before sexual maturation. Although the scallops deriving from different stocks are often cultured in the same sea area, no mixing of the stocks has occurred. Also, natural populations of bay scallop have been not found in China (personal observation). Therefore, gene flow between different stocks is believed to be limited. The two stocks of bay scallop in China may represent different genetic stocks. When genetic improvement programs are planned, the design should be based not only on the distinction of available stocks, but also on the genetic characteristics of important production traits at different life stages during

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hatchery and field grow-out phases (Cruz & Ibarra 1997). In China, the bay scallop has been exclusively produced in hatcheries for more than two decades, and hatchery production of seed provides an opportunity for genetic improvement.

To determine if heterosis exists and can be used for genetic improvement in the bay scallop, four groups including two reciprocal hybrid crosses and their parental controls were produced by pair-mating between two stocks and mass-spawning within each stock. The four groups were reared under the same conditions to minimize effects of environmental factors. Heterosis was estimated and compared for several traits at different life stages, including hatching success of fertilized-eggs, larval survival, and growth at larvae, spat and adult stages.

## MATERIAL AND METHODS

### Parental Stocks and Conditioning

The two parental stocks of bay scallops used in this study, named "A" and "B", were the same age and had been cultured under the same environmental conditions in the Laizhou Bay of Bohai Sea, but they differed in their origin and breeding history. Stock A is derived from the first introduction from Connecticut, USA in 1982 and has been successively cultured in China for more than 20 generations. Stock B is the forth generation from a recent introduction from Virginia and Massachusetts USA in 1999. The two parental stocks used in the present study were produced as the control lines (nonselected) for a selection experiment in 2002 (Zheng et al. 2004a). In March 2003, shell lengths of a random sample of 200 individuals from each stock were measured with a vernier caliper (accuracy, 0.02 mm), and 500 individuals of the two stocks were conditioned using the method described by Zheng et al. (2004a). In short, scallops of two stocks were placed separately in lantern nets (8 layers/net and 20 scallops/layer) and conditioned in a 50 m<sup>3</sup> concrete tank. Each scallop was fed with  $4 \times 10^6$  cells of *Nitzschia closterium* daily. Water temperature was raised from 7–8°C to 16°C, and kept at 16°C for a week. Next, temperature was gradually raised from 16°C to 18°C, and kept at 18°C thereafter. Salinity ranged from 30–31 ppt. Water was changed and feces removed by siphoning from the bottom of the tank daily. After approximately one week at 18°C, most of the breeders were ripe or reached ripeness stage IV, as visually determined according to Sastry (1963).

### Experimental Design and Treatments

On May 4, 10 sexually mature scallops with gonadal condition at stage IV (Sastry 1963) were chosen from each stock for spawning. To induce spawning, the combined method of by injecting serotonin into scallop's adductor muscle (Cruz & Ibarra 1997) and thermal shock by raising water temperature from 18°C to 23°C was used, and the following procedure was able to prevent self-fertilization. First, each scallop was injected with 0.1 mL of 0.02 mM serotonin (5-hydroxytryptamine, Sigma) into the adductor muscle. Next, each spawner was separately placed in one 5-L polyethylene bucket containing 3-L of 23°C seawater (filtered to 30 µm). After 15–20 min, all animals began to release sperms. Each spawner released sperm several times during a 1-h period. Then, each spawner was taken out and rinsed 2–3 times with 23°C seawater and individually placed in one 5-L bucket containing 3-L filtered seawater at 23°C. In general, most individuals began to release eggs after about 20 min. Each spawner released eggs in 2–3

expulsions during approximately 30 min. After spawning, eggs were washed on a 30-µm screen to remove sperm if any and placed into one 40-L bucket, respectively. Fecundity was estimated by counting the number of eggs from each scallop. The diameter of 20 eggs from each spawner was measured under microscope (×100). Eggs were sampled and double-checked under microscope for fertilization caused by sperm contamination, and eggs were discarded if they were contaminated.

Eggs of each female were divided in two parts, one was pair-crossed to a male from the other stock, and the other part was pooled first within one stock and then mass-crossed with mixed sperm from the same stock. Therefore, four distinct groups were made and used in the study, including two reciprocal hybrid crosses AB (A♀ × B♂) and BA (B♀ × A♂) produced by pair-crossing between two stocks and two parental groups AA (A♀ × A♂) and BB (B♀ × B♂) produced by mass-crossing within each stock. Fertilized eggs were placed in polyethylene buckets for incubation. Twenty reciprocal-crosses were placed respectively in twenty 40-L buckets, and each parental group was separated into three replicates, and then each replicate was placed in one 80-L bucket. Water temperature and salinity during incubation were 23°C and 30 ppt, respectively.

Thirty hours after fertilization, D-stage larvae hatched, and hatching success of fertilized-egg was estimated by counting the number of normal D-stage larvae per replicate. Afterward, the larvae from the same reciprocal cross group were collected from each bucket using a 30-µm sieve, placed mixed in one 80-L polyethylene bucket with seawater and divided into three parts equally, conforming 3 replicates as with the parental stocks. Initial stocking density was 10 larvae mL<sup>-1</sup> for all groups.

To minimize environmental effects, all four groups were reared under the same condition during larval culture, spat nursery and adult grow-out according to methods described by Zheng et al. (2004a). No culling of small individuals was conducted at any stage, therefore reducing potential effects of altering the evaluated traits.

### Trait Assays

Four traits were measured: (1) parental fecundity, measured as the average number of eggs spawned by each spawner; (2) hatching success, measured as the ratio of the number of normal D-stage larvae to the number of fertilized eggs; (3) larval survival, as the ratio of the numbers of viable larvae at culture days 4, 7, and 10 to the numbers of larvae on day 1; and (4) growth, measured as shell length at larval, spat, and adult life stages.

The numbers of eggs, normal larvae at day 1, and larvae at days 4, 7 and 10 were estimated as the average of three samples of 1 mL each using a dissecting microscope (×20), extrapolating numbers then to total volume. Shell length (the longest anterior-posterior distance) of 30 randomly taken individuals per replicate was measured using different methods, depending on the life stage. Larvae were killed with 4% formaldehyde, and measured on days 1, 4, 7 and 10 after using a microscope (×100) equipped with an ocular micrometer. Spats were measured alive on days 20, 30, 40 and 50 using a dissecting microscope (×20–40) equipped with an ocular micrometer. Adults were also measured alive at days 70, 100, 130 and 160 using a Vernier caliper (accuracy: 0.02 mm).

### Estimate of Heterosis

Because scallops in commercial hatcheries have been consistently produced by the method of mass spawning, the two mass-

crossed groups (AA and BB) can be considered as a representative sample of the two populations to estimate heterosis in this study.

In this study, midparent heterosis ( $H_{MP}$ ) is defined as the difference between the mean of the reciprocal hybrid crosses and the mean of the two parents (Falconer 1981) and calculated by the following equation:

$$H_{MP}\% = \frac{FC - P}{P} \times 100$$

where,  $FC$  = average phenotypic value of the reciprocal crosses,  $P$  = average phenotypic value of two parental populations.

Additionally, single-parent heterosis (genetic gain or improvement) is defined as the proportional increment in the phenotypic values of single-parent stock caused by crossing and calculated by the following equation (Cruz & Ibarra 1997):

$$H_X\% = \frac{FC - P(X)}{P(X)} \times 100$$

where,  $H_X$  = single-parent heterosis (genetic gain or improvement) of stock  $X$ ;  $P(X)$  = phenotypic value of parental stock  $X$ ;  $FC$  as defined before.

#### Statistical Analyses

Differences between parental stocks in fecundity, egg size (diameter) and shell length, as well as single-parent heterosis (genetic gain or improvement) for each stock were tested using the student  $t$ -test. Differences in hatching success, larval survival and growth among the four groups and in heterosis among different life history stages, ages, and types (midparent heterosis  $H_{MP}$ , single-parent heterosis for stock A  $H_A$ , and single-parent heterosis for stock B  $H_B$ ) were analyzed by multiple comparisons of means using a 1-way ANOVA. Shell length when appropriate was converted to logarithms to increase normality and homoscedasticity (Neter et al. 1985). Because data on hatching success, larval survival and heterosis were in percentages, they were transformed to arcsine before analysis (Rohlf & Sokal 1981). Analyses were done using SPSS (Statistical Program for Social Sciences) 11.5 software for Windows. Significance level for all analyses was set to  $P < 0.05$  unless noted otherwise.

## RESULTS

#### Differences Between the Two Parental Stocks

The two parental stocks were significantly different in body size and fecundity but not in egg size (Table 1). Body size of stock B as measured in shell height was 7% larger than that of stock A. Fecundity of stock B was 1.07 times higher than that of stock A.

TABLE 1.

Parental shell size, fecundity and egg size (diameter) of two stocks of *Argopecten irradians irradians*. Standard deviation and samples size are given in parenthesis.

Stock	Parental Size (mm) <sup>1</sup>	Fecundity (×10 <sup>4</sup> eggs/spawner)	Egg Size (μm)
A	51.5 <sup>a</sup> (5.3, 200)	78.0 <sup>a</sup> (17.9, 10)	58.0 <sup>a</sup> (2.8, 200)
B	54.9 <sup>b</sup> (10.4, 200)	161.1 <sup>b</sup> (62.2, 10)	58.3 <sup>a</sup> (3.2, 200)

<sup>1</sup> Within each column, means with the same letter are not statistically different ( $P > 0.05$ ).

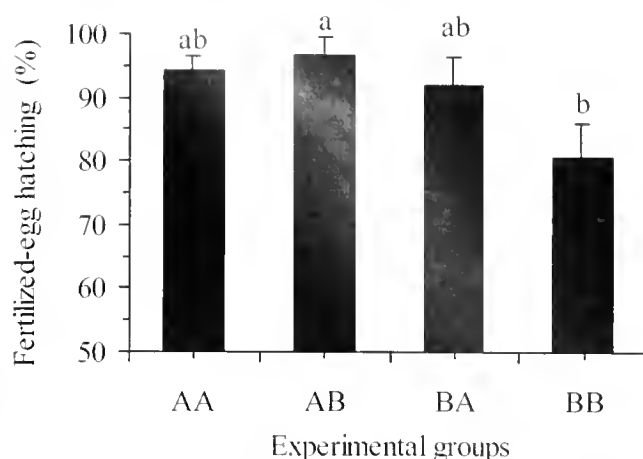


Figure 1. Hatching success of the four genetic groups of *Argopecten irradians irradians*. AA and BB are the two parental groups ( $A \times A$  and  $B \times B$ ), AB and BA are the reciprocal hybrid crosses ( $A \times B$  and  $B \times A$ ). Error bars are  $\pm 1$  SD. Means with the same letter above error bars are not statistically different ( $P > 0.05$ ).

#### Hatching

Hatching success of the hybrid cross AB was the highest, although it did not differ significantly from that of AA or the reciprocal hybrid cross BA (Fig. 1). The lowest hatching success was seen in the parental BB stock, but the differences among BB, AA and reciprocal hybrid cross BA was not significant. The difference between the best (AB) and worst (BB) performing groups was significant. Midparent heterosis for hatching success ( $H_{MP}$  = 7.9%) fell between single-parent heterosis A ( $H_A$  = 10.6%) and B ( $H_B$  = 5.3%), all were significantly bigger than zero ( $P < 0.01$ ).  $H_A$  was significantly bigger than  $H_B$  ( $P < 0.01$ ) (Table 2).

#### Larval Survival

No significant differences ( $P > 0.05$ ) for larval survival existed among four groups at days 4 and 7, but survival of hybrid AB were significantly higher than that of randomly mated BB ( $P < 0.05$ ) at days 10 (Fig. 2). Midparent heterosis ( $H_{MP}$ ), single-parent heterosis A ( $H_A$ ) and single-parent heterosis B ( $H_B$ ) for larval survival were presented in Table 2. All heterosis was positive, and no significant differences were observed among them at the same age. Both  $H_{MP}$  and  $H_A$  increased significantly ( $P < 0.05$ ) with increas-

TABLE 2.

Heterosis (%) for hatching success and larval survival in *Argopecten irradians irradians* at days 4, 7 and 10.  $H_{MP}$  is the mid-parent heterosis,  $H_A$  and  $H_B$  are the single-parent heterosis of stock A and B, respectively.

Heterosis	Hatching Success <sup>1</sup>	Larval Survival <sup>1</sup>		
		Day 4	Day 7	Day 10
$H_{MP}$ (%)	7.9 <sup>ab</sup> (3.6)	6.0 <sup>a</sup> (3.0)	11.2 <sup>ab</sup> (3.7)	15.1 <sup>b</sup> (1.9)
$H_A$ (%)	10.6 <sup>a</sup> (3.3)	2.8 <sup>a</sup> (7.2)	12.3 <sup>ab</sup> (3.2)	17.3 <sup>b</sup> (3.3)
$H_B$ (%)	5.3 <sup>b</sup> (4.8)	9.2 <sup>a</sup> (1.4)	10.2 <sup>a</sup> (4.9)	12.8 <sup>ab</sup> (5.1)

<sup>1</sup> Means that share the same letter within a column (for hatching success) or row (for larval survival) are not statistically different ( $P > 0.05$ ).

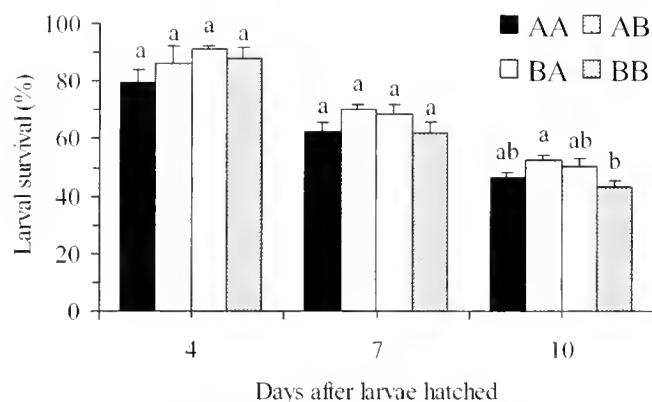


Figure 2. Larval survival of the four genetic groups of *Argopecten irradians irradians* at Day 4, 7 and 10. AA and BB are the two parental groups ( $A \text{♀} \times A \text{♂}$  and  $B \text{♀} \times B \text{♂}$ ). AB and BA are the reciprocal hybrid crosses ( $A \text{♀} \times B \text{♂}$  and  $B \text{♀} \times A \text{♂}$ ). Error bars are  $\pm 1$  SD. At the same larval age, means with the same letter above error bars are not statistically different ( $P > 0.05$ ).

ing age, although  $H_B$  showed no significant increase ( $P > 0.05$ ).  $H_{MP}$ ,  $H_A$  and  $H_B$  were all significantly bigger than zero ( $P < 0.01$ ), which were 10.8%, 10.8% and 10.7%, respectively.

#### Growth

Shell length of the two parental stocks was significantly different from day 4 on, with the shell length of the BB stock always being larger than the AA stock (Table 3). Shell length of both hybrid crosses were intermediate between the two parental stocks and significantly larger than the shell length of the AA stock from day 7 to the end of the experiment (days 160). The BA cross was significantly larger than the AB cross and as large as the BB stock on days 4 and 30.

Mid-parent heterosis ( $H_{MP}$ ) for growth clearly fell between two single-parent heterosis and increased with age (Fig. 3).  $H_{MP}$  at the larval stage (3.0%) was significantly smaller than that at spat (8.4%) ( $P < 0.05$ ) and adult (10.1%) stages ( $P < 0.01$ ) (Table 4).

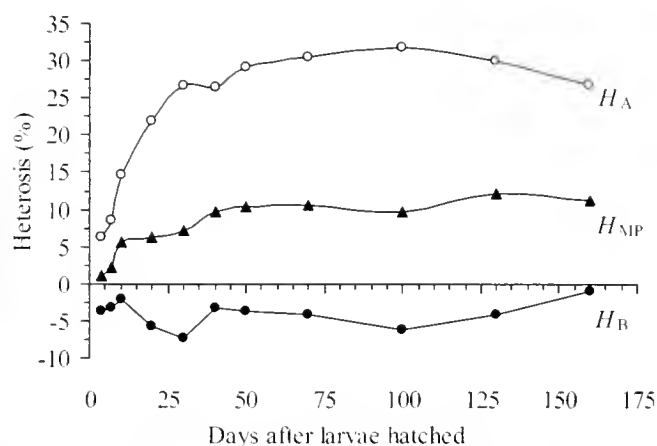


Figure 3. Heterosis for growth at different ages after hatching in *Argopecten irradians irradians*.  $H_{MP}$  is the midparent heterosis.  $H_A$  and  $H_B$  represent single-parent heterosis of stock A and B, respectively.

For single-parent heterosis, positive values were observed for stock A throughout the studying period, whereas negative values were observed for stock B. Analysis of variance further demonstrated that the average of single-parent heterosis of stock A (23.0%) was significantly bigger than that of stock B (−4.0%) ( $P < 0.001$ ,  $t = 9.221$ ,  $df = 10$ ). Moreover, single-parent heterosis of stock A varied significantly among the three life stages (Table 4), ranging from 9.8% at larval stage, to 29.8% at adult stages.

#### DISCUSSION

This study provides two major observations on heterosis in bay scallops. First, crosses between the two evaluated stocks of *A. irradians irradians* revealed midparent heterosis for all traits, although in no case where heterosis was useful (i.e., the hybrid crosses outperformed both parental stocks). Secondly, the magnitude of heterosis was not constant, but varied among traits and life history stages; and different traits exhibited different single-parent heterosis.

TABLE 3.

Shell length (SD) of the four genetic groups in *Argopecten irradians irradians* at different ages. AA and BB are the two parental groups ( $A \text{♀} \times B \text{♂}$  and  $B \text{♀} \times A \text{♂}$ ).

Stage and Age	Experimental Genetic Groups <sup>1</sup>			
	AA	AB	BA	BB
Larvae (μm)				
1	90.1 <sup>a</sup> (4.3)	91.0 <sup>a</sup> (2.9)	90.8 <sup>a</sup> (3.6)	91.5 <sup>a</sup> (4.0)
4	112.3 <sup>a</sup> (8.9)	116.3 <sup>a</sup> (7.9)	122.3 <sup>b</sup> (7.5)	124.0 <sup>b</sup> (11.4)
7	131.0 <sup>a</sup> (12.3)	139.3 <sup>b</sup> (10.0)	145.1 <sup>bc</sup> (11.7)	147.0 <sup>c</sup> (18.1)
10	148.8 <sup>a</sup> (15.9)	168.5 <sup>b</sup> (17.4)	172.7 <sup>b</sup> (16.6)	174.3 <sup>b</sup> (22.14)
Spat (mm)				
20	0.241 <sup>a</sup> (0.039)	0.287 <sup>b</sup> (0.053)	0.301 <sup>b</sup> (0.055)	0.312 <sup>b</sup> (0.072)
30	0.637 <sup>a</sup> (0.142)	0.760 <sup>b</sup> (0.112)	0.854 <sup>c</sup> (0.092)	0.870 <sup>c</sup> (0.158)
40	1.932 <sup>a</sup> (0.348)	2.392 <sup>c</sup> (0.346)	2.494 <sup>c</sup> (0.344)	2.524 <sup>c</sup> (0.483)
50	3.923 <sup>a</sup> (0.794)	4.997 <sup>b</sup> (0.808)	5.134 <sup>b</sup> (0.718)	5.255 <sup>b</sup> (1.120)
Adult (mm)				
70	11.12 <sup>a</sup> (2.78)	14.34 <sup>b</sup> (2.42)	14.70 <sup>b</sup> (2.16)	15.13 <sup>b</sup> (2.86)
100	20.02 <sup>a</sup> (2.80)	25.44 <sup>b</sup> (2.95)	27.33 <sup>bc</sup> (2.92)	28.13 <sup>c</sup> (3.66)
130	29.78 <sup>a</sup> (5.33)	38.02 <sup>b</sup> (3.64)	39.43 <sup>b</sup> (3.60)	40.35 <sup>b</sup> (6.05)
160	39.42 <sup>a</sup> (6.21)	48.49 <sup>b</sup> (5.79)	51.46 <sup>b</sup> (4.89)	50.45 <sup>b</sup> (8.19)

<sup>1</sup> Within each row, means with the same letter are not statistically different ( $P > 0.05$ ).

TABLE 4.

Average heterosis (SD) for growth in *Argopecten irradians irradians* during three different life stages.  $H_{MP}$  is the mid-parent heterosis.  $H_A$  and  $H_B$  are single-parent heterosis for stock A and B, respectively.

Heterosis	Stage		
	Larvae	Spat	Adult
$H_{MP}$ (%)	3.0 <sup>ab</sup> (2.8)	8.4 <sup>c</sup> (1.9)	10.9 <sup>c</sup> (1.1)
$H_A$ (%)	9.8 <sup>d</sup> (4.3)	26.1 <sup>d</sup> (3.0)	29.8 <sup>d</sup> (2.1)
$H_B$ (%)	-3.0 <sup>a</sup> (0.9)	-5.0 <sup>b</sup> (1.9)	-3.8 <sup>b</sup> (2.2)

<sup>1</sup> Within each row or line, means with the same letter are not statistically different ( $P > 0.05$ ).

The observed differences in parental size and fecundity between stock A and B (Table 1) imply that there may be genetic differences between these stocks. Two reasons can potentially explain such results. First, the two parental stocks, A and B, have different origin and breeding history. The breeding history of stock A includes its reproduction under artificial conditions for 16 more generations than stock B. Because of this, it is expected that stock A has experienced a stronger accumulation of inbreeding, whereas stock B might be less inbred. Second, the origin of stock A and stock B are also different, with the former being derived from only 26 individuals (Zhang et al. 1986), whereas the later one was derived from 406 individuals, including 200 of wild origin and 206 from a one generation in captivity population (Gu, Z.—one of the introducers, personal communication). Because of the small initial number of stock A and the mass spawning for so many generations, it is expected that stock A has accumulated high levels of inbreeding that is affecting fitness-related traits. The molecular evidence of reduced genetic diversity in stock A was provided by comparing with other natural populations (Blake et al. 1997). Stock B, however, may have retained high genetic diversity because of its shorter breeding history and larger initial population size.

Crosses between genetically differentiated subpopulations are expected to increase heterozygosity, reduce effects of recessive lethal genes and enhance fitness, resulting in heterosis or hybrid vigor (Whitlock et al. 2000). The hybrid crosses in this study exhibited positive midparent heterosis for hatching success, larval survival and growth. The presence of heterosis (positive or negative) has been reported for other marine bivalves (Mallet & Haley 1983, Hedgecock et al. 1995, 1996, Bayne et al. 1999) and other hermaphroditic pectinid species (Cruz & Ibarra 1997, Cruz et al. 1998, Beaumont et al. 2004). In the present study, the means for the hybrid crosses for all traits were always higher than that of the parental stocks, indicating a positive association between heterozygosity and the traits. Sheridan (1981) stated that the positive association is a common consequence of crossbreeding. Classical quantitative genetic studies of crossbreds produced by crossing inbred lines have uncovered remarkable heterosis in growth and its physiological components at larval, juvenile and adult stages and has implicated epistasis as a significant cause of this heterosis (Hedgecock et al. 1996).

In this study, we found the magnitude of heterosis is not constant but varies among traits and life stages. Midparental heterosis for fitness-related traits such as hatching success (7.9%) and larval survival (10.8%) is bigger than that for morphological traits such as larval shell length (3.0%). Similar results have previously been obtained by comparing pair-crosses between the two stocks with their self-fertilization in this species (Zheng et al. 2004b), and heterosis for larval survival (32.9%) was bigger than that for larval shell length (8.5%) at days 7. Why fitness-related traits such as survival exhibit greater heterosis than morphological traits? It is known that heterosis depends on the presence of directional dominance for the loci involved in the specific trait (Falconer 1981, Lamkey & Edwards 1998, 1999), and the coefficient of dominance variance is larger in fitness-related traits than in morphological traits (Roff 1998). Then, as stated by Lynch and Walsh (1998), fitness-related traits are expected to present directional dominance because mutations affecting those traits are typically deleterious and recessive. DeRose and Roff (1999) also emphasized that morphological traits exhibit little or no directional dominance. It is also known that life-stage specific difference in inbreeding depression are common and may depend on environmental, developmental or genetic factors (Husband & Schemske 1996). Therefore, complementary to the phenomenon of inbreeding depression, it is not uncommon for heterosis to be life-stage specific, as seen in this study, where midparent heterosis for growth was 3.0% at larval stage, 8.4% at juvenile stage and 10.1% at adult stage. It is also possible that heterosis is masked by maternal effects at early stages. Similar results have been reported in other marine bivalves. For example, in hard clam *Mercuraria mercenaria*, crossbred offspring were not consistently faster growing than purebred offspring (Manzi et al. 1991). In catarina scallop *A. circularis*, heterosis values for larval growth were 0 at day 11 and 6.8% at day 17 (Cruz & Ibarra 1997), and over 10% at adult stages (Cruz et al. 1998). In the Pacific oyster *Crassostrea gigas*, heterosis for body size were 1.0% at day 2 and 7.7% at day 340 (Hedgecock et al. 1995).

Finally, we found single-parent heterosis for growth between the two stocks was significantly different. The difference in single-parent heterosis (genetic gain or improvement) for growth between the two stocks could be caused by genetic differences between the two parental stocks. For stock A, performance may have been depressed by inbreeding and improved by crossing with stock B with higher genetic diversity; thus the value of single-parent heterosis (genetic gain or improvement) for growth is always positive. For stock B, there is little or no inbreeding depression, and the stock is performing better than stock A; thus the value of single-parent heterosis (genetic gain or improvement) for growth is negative.

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## SEDIMENT SELECTION BY JUVENILE SEA SCALLOPS (*PLACOPECTEN MAGELLANICUS* (GMELIN)), SEA STARS (*ASTERIAS VULGARIS* VERRILL) AND ROCK CRABS (*CANCER IRRORATUS* SAY)

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**ABSTRACT** We examined sediment selection by juvenile sea scallops (*Placopecten magellanicus*) and their predators, sea stars (*Asterias vulgaris*) and rock crabs (*Cancer irroratus*). In laboratory trials, groups of scallops (~30 mm shell height) were simultaneously offered four sediment types in the presence and absence of a sea star or a rock crab: (1) glass representing a homogeneous, hard bottom; (2) sand; (3) granule and (4) pebble. As well, individual predators were offered the four sediment types without scallops. The number of scallops and the proportion of time predators spent on each sediment type were monitored over time. When compared with the expected distribution, scallops avoided glass and tended to select granule and pebble sediments when alone. In the presence of a rock crab, scallops also avoided glass. However, scallop distribution was similar to the expected distribution when a sea star was present, because sea star encounters are an important trigger of scallop swimming, leading to frequent redistribution of scallops. Sea stars spent less time than expected on glass, whereas crabs spent more time than expected on sand. For both predators, distribution did not change significantly in the presence or absence of scallops. In sum, scallop distribution appears more dependent on predator distribution than the reverse. Predation of scallops by sea stars and rock crabs was not influenced by sediment type. Our results have implications for the bottom culture of scallops. Because scallops select heterogeneous sediments over homogeneous ones, dispersal of scallops may be important on unsuitable sediments. Also, dispersal may be higher when sea stars are present at an aquaculture site.

**KEY WORDS:** bottom culture; distribution; rock crab, *Cancer irroratus*; sea scallop, *Placopecten magellanicus*; sea star, *Asterias vulgaris*; sediment type; selection; substrate

### INTRODUCTION

The bottom culture of sea scallops (*Placopecten magellanicus*), where juvenile scallops are released (seeded) onto the sea bottom and allowed to grow to commercial size, has gained considerable interest in the last two decades on the coast of the northwest Atlantic (Couturier et al. 1995). However, the success of this type of scallop aquaculture has been limited because large portions of seeded scallops (40% to 99%) often do not survive (Barbeau et al. 1996, Hatcher et al. 1996, Nadeau & Cliche 2004). The two main processes influencing loss of seeded scallops are predator-related mortality (specifically by predatory sea stars *Asterias* spp. and rock crabs *Cancer irroratus* in coastal areas of Atlantic Canada), and dispersal of seeded scallops away from the culture site (Barbeau et al. 1996, Wong et al. 2005). Both of these processes can be influenced by the physical characteristics of the bottom culture site, such as sediment type, water temperature and current velocity. In this study, we focus on sediment type. Certain sediment types may reduce a predator's searching and handling ability of prey, or provide prey with physical refuges that reduce detection by predators (Sponaugle & Lawton 1990, Arseneault & Himmelman 1996). Additionally, predators and prey may disperse from habitats where sediments interfere with feeding behaviors, chemoreception, or general movement (Sponaugle & Lawton 1990). Previously, Wong and Barbeau (2003) examined the effects of sediment type on predator-prey interactions when sea stars and rock crabs preyed on juvenile sea scallops. This previous study, while directly investigating effects of sediment type on predation, did not examine choice of sediment by predators or prey. If offered a number of sediment types simultaneously, sea stars, rock crabs and juvenile scallops may select a particular sediment type to which they will disperse and spend the majority of time there.

Field observations of sea star, rock crab and scallop abundances in a variety of habitats suggest that these animals select specific sediments. Juvenile sea scallops (*P. magellanicus*) (<50 mm shell height, SH), although rarely observed in the field, have been observed on smaller gravel sediments (2.00–15.00 mm diameter; Thouzeau et al. 1991), gravel and shells embedded in a silt matrix (Barbeau et al. 1996, Wong et al. 2005) or attached to substrates that provide vertical relief, such as hydrozoan branches or amphipod tubes (Larsen & Lee 1978). Adult sea scallops (≥50 mm shell height) are found on a variety of sediment types, but they are more commonly found on gravel (2.00–64.00 mm diameter) or gravelly-sand (0.0625–16.00 mm diameter) sediments (Langton & Uzmann 1989, Langton & Robinson 1990, Stokesbury & Himmelman 1995, Stokesbury 2002, Wong et al. 2005). Sea stars (*Asterias vulgaris*) and rock crabs (*C. irroratus*) are often observed on the same sediment types as adult and juvenile sea scallops (Wong et al. 2005). Sea stars tend to be more abundant on coarser sediments (Langton & Uzmann 1989, Himmelman & Dutil 1991), but are found on most sediment types ranging from mud to boulders (0.06 µm–1024 mm). Rock crabs are also common on all sediment types (Scarratt & Lowe 1972, Bigford 1979, Drummond-Davis et al., 1982); those found on sand (0.0625–2.00 mm diameter) are often wholly or partially buried (Scarratt & Lowe 1972).

Dispersal from one sediment type to another requires a well-developed locomotory ability. Juvenile sea scallops are able to actively disperse and potentially select specific sediment types by swimming. Rapid clapping of the valves causes enclosed water to be forced out near the shell hinge and the scallop is propelled forward by jet propulsion. Scallops 11–80 mm in shell height (SH) are efficient swimmers and capable of extended horizontal flight (~0.5–3 m) (Dadswell & Weihs 1990, Carsen et al. 1996). Smaller scallops (<11 mm SH) tend to swim vertically up into the water column with little horizontal displacement (Manuel & Dadswell 1991). Large adult scallops (>100 mm SH) are limited by heavy shells, and can only shuffle for short distances along the seabed (Dadswell & Weihs 1990). Sea stars and rock crabs are highly

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mobile and likely have little difficulty dispersing to certain sediment types. Sea stars use tube feet to travel at moderate velocities ( $\sim 2 \text{ cm min}^{-1}$ ), whereas rock crabs use four pairs of walking legs to travel at relatively high velocities ( $\sim 66 \text{ cm min}^{-1}$ ) (Barbeau et al. 1994).

Sediment selection by juvenile sea scallops, sea stars and rock crabs is likely to have important consequences for the survival of seeded scallops during bottom culture. For example, seeding scallops onto a sediment type that enhances filter feeding and is avoided by sea stars and crabs may increase survival of scallops. The objective of our study was to determine the sediment selection by juvenile sea scallops, sea stars and rock crabs when offered four different sediment types simultaneously. A laboratory experiment was conducted in which the distribution of scallops on the four sediment types when predators were present and absent was quantified over time. Additionally, the proportion of time sea stars and rock crabs spent on each sediment type in the presence and absence of scallops was quantified.

## MATERIALS AND METHODS

### Experimental Materials

Sediment selection by juvenile sea scallops (*Placopecten magellanicus*), sea stars (*Asterias vulgaris*) and rock crabs (*Cancer irroratus*) was investigated from 29 July to 13 August 2001, using a laboratory experiment at the Huxman Marine Science Center, St. Andrews, New Brunswick, Canada. Fiberglass circular tanks (measuring 120 cm in diameter and 27 cm high) were used. Flow-through sea water was sand filtered to  $20 \mu\text{m}$  and regulated between  $0.6\text{--}2 \text{ L min}^{-1}$ , the light regimen was 14 h light: 10 h dark, and water temperature ranged between  $12^\circ\text{C}$  and  $14^\circ\text{C}$ .

Juvenile sea scallops were obtained from Sea Perfect Cultivated Products, Arichat, Nova Scotia. They were held in 260 L tanks for six days before the experiment began. Scallops were fed algae paste (Innovative Aquaculture Products Ltd) prior to and during the experiments at a concentration of  $1.0 \times 10^4 \text{ cells mL}^{-1}$  of water (Hollett & Dabinnett 1989). Scallops were  $31.2 \pm 3.9 \text{ mm}$  (mean  $\pm$  S.D.,  $n = 30$ ) in shell height (SH), measured as the distance between the middle of the dorsal hinge to the furthest ventral edge of the shell.

All predators originated from Passamaquoddy Bay in the Bay of Fundy, New Brunswick. Sea stars were collected by trawling, and were held in small tanks (measuring 60 cm long  $\times$  30 cm wide  $\times$  30 cm high) for 3 wk before the experiments began. Only intact sea stars with all 5 arms were used. Sea stars were  $213.5 \pm 35.3 \text{ mm}$  (mean  $\pm$  S.D.,  $n = 16$ ) in diameter, measured as the distance between the tip of an average sized arm to the middle of the oral region, multiplied by two. Rock crabs were obtained by trawling or from local crab fishers, and were held in separate small tanks for 3 wk before the experiment began. Only male crabs with both chelae and all walking legs were used. Crabs were  $108.4 \pm 7.0 \text{ mm}$  (mean  $\pm$  S.D.,  $n = 16$ ) in carapace width (CW), measured as the distance between the two most distal marginal teeth. To standardize hunger level, both predator species were fed blue mussels until 6 d prior to the experiment. Animals were only used once during the experimental trials.

### Experimental Design

Trials of 12-h duration were conducted in which groups of scallops and/or individual sea stars and rock crabs were placed in

tanks with four different sediment types: glass, sand, granule and pebble. Glass consisted of a flat, 0.625-cm thick Lexan sheet, and represented a homogeneous, hard substrate. Sand consisted of 21% granule and 79% sand, and granule was 12% sand, 29% pebble and 59% granule. Pebble was uniform with no sand or granule present, and ranged from  $30 \times 10 \text{ mm}$  to  $75 \times 35 \text{ mm}$  in particle size (Wong & Barbeau 2003). These sediment classifications follow Wentworth scale of particle sizes, where sand is  $0.0625\text{--}2.00 \text{ mm}$ , granule  $2.01\text{--}4.00 \text{ mm}$ , and pebble  $4.01\text{--}64 \text{ mm}$  in diameter (Wentworth 1922, Folk 1974). Sediments were allowed to soak overnight in seawater before each trial to allow a biofilm to accumulate. For each trial, sediment types were placed into specific, randomly chosen pie-shaped sections that prevented mixing of the sediment types using a separation apparatus (Wright 2002). Sediments were  $\sim 15 \text{ cm}$  in depth. Replicates of the following treatments were randomly allocated into the tanks: scallops alone (group of 40), scallops with one sea star, scallops with one rock crab, one sea star alone, and one rock crab alone. Eight replicates of each treatment were conducted, for a total of 40 trials. In trials with a sea star or crab, the predator was placed on a randomly chosen sediment 12 h prior to the beginning of the trial. In trials with scallops, 10 scallops were released onto each sediment near the center of the tank and allowed to acclimate for 5 min before the start of the trial.

### Collection of Data

To determine sediment selection by scallops, the number of scallops on each sediment type was noted at hourly intervals during the 12-h trial. For sea stars and rock crabs, the time spent on each sediment type was quantified during five continuous 1-h observation periods, randomly chosen during the 12-h trial. The proportion of time predators spent on each sediment type was calculated as the total time spent on one sediment type divided by the total observation time. Animals were considered on a certain sediment type when  $\geq 50\%$  of the body surface was in contact with that sediment. Sea stars were observed on the walls of the tank, and so this was designated as a fifth substrate ("other") in trials involving sea stars. If a scallop was consumed, it was immediately replaced in the center of the sediment type on which consumption took place.

### Statistical Analyses

Scallop and predator data were analyzed separately. Sediment selection by scallops was investigated using two methods. In the first scallop analysis, a one-sample Hotelling  $T^2$  test was conducted to determine if scallop distribution at the end of the trial (720 min) differed from the expected distribution of 10 scallops on each sediment type.  $F$  values were calculated as:

$$F = \frac{n-p}{p(n-1)} T^2,$$

where  $n$  = number of replicates,  $p$  = number of sediment types and  $T^2$  = calculated Hotelling  $T^2$  statistic (Johnson & Wichern 1998). A separate Hotelling  $T^2$  test was conducted for scallops alone, scallops with sea stars and scallops with rock crabs. If scallop distribution differed significantly from the expected distribution, 95% confidence intervals were calculated for each sediment type by:



$$\bar{x}_i \pm \left[ (1/n) s_i^2 \frac{(n-1)p}{n-p} F_{\alpha, p, n-p} \right]^{1/2},$$

where  $\bar{x}_i$  = mean number of scallops on sediment type  $i$ , and  $s_i^2$  = sample variance for sediment type  $i$  (Johnson & Wichern 1998). A sediment type contributed to the significant result when the confidence interval did not include the expected value (Roa 1992).

In the second scallop analysis, scallop distribution over time in the presence and absence of predators was analyzed using a split-plot multivariate analysis of variance (MANOVA), with predator presence (3 levels: no predator present, sea star present, crab present) as a fixed factor, time (13 levels from 0–12 h) as the split-plot factor, and tank (8 replicate tanks) as the random plot factor. Significant results were identified using Pillai's trace. This is a robust statistic that is not influenced by violation of multivariate normality, which could have resulted from our relatively small sample size (Scheiner 2001). When significant results were obtained, we conducted canonical analyses for the significant factors. We observed the squared canonical correlation of each canonical variate generated to determine the percentage of variance explained by the model. We also observed the magnitude and sign of the coefficients of the first standardized canonical variate to aid interpretation of significant main effects. Similar signs of coefficients indicate that dependent variables are positively correlated across treatments; in other words, selection for sediment changes in a similar direction across treatments. Magnitude of values indicates the relative contribution of each sediment type to the significant result (Scheiner 2001).

Sediment selection by predators was also investigated using two methods. In the first predator analysis, one-sample Hotelling  $T^2$  tests were used to determine if the proportion of time predators spent on each sediment type was different from the expected distribution (i.e., sea star: 0.126 on each of the four sediments and 0.496 on "other"; rock crab: 0.25 on each sediment; calculated by dividing the area covered by the substrate by the total area available to the predator). Separate tests were conducted for predators alone and in the presence of scallops. In the second predator analysis, two-sample Hotelling  $T^2$  tests were used to compare the proportion of time predators spent on each sediment type when scallops were present and when scallops were absent.  $F$  values for two-sample tests were calculated as:

$$F = \frac{(n_1 + n_2 - p - 1)T^2}{(n_1 + n_2 - 2)p}.$$

Significant results in above-mentioned Hotelling  $T^2$  tests were investigated by calculating 95% confidence intervals as described for the scallop analyses. A two-sample  $t$ -test was also used to compare the proportion of time sea stars spent on vertical surfaces (i.e., "other" substrate) in the presence and absence of scallops.

One-sample Hotelling  $T^2$  tests were also used to determine if predation rate on scallops on each sediment type by predators was different from the expected predation rate. Expected predation rate on each sediment type took into account the distribution of scallops; therefore, expected predation rate for a particular sediment type was calculated by multiplying the sum of observed predation rates over all sediment types (averaged over the 8 replicates) by the proportion of scallops on that sediment type at 720 min (averaged over the 8 replicates).

In most Hotelling  $T^2$  tests conducted, linear dependence between columns of the data matrix existed because the data were restricted to total to 40 scallops (when analyzing distribution of

scallops) or to 1 (when analyzing proportion of time predators spent on each sediment type). This linear dependence leads to an undefined inverse covariance matrix and the Hotelling  $T^2$  statistic cannot be calculated (see Johnson & Wichern 1998 for matrix algebra). To alleviate this problem, we removed data for one sediment type that was similar to another sediment type (see Table captions for the exact data removed). Data were not removed from analyses of scallop distribution when sea stars and crabs were present, because some scallops were consumed and data were not restricted to total to 40. The assumption that data came from a normal multivariate population was tested using probability plots for data on each sediment type (Roa 1992). This assumption was not violated for any case. The assumption of equal covariance matrices between groups for two-sample Hotelling  $T^2$  tests were tested using Cochran test for data on each sediment. For the MANOVA, equal covariance was determined when the sign of the correlation between dependent variables at each level of the treatment variables were the same (Scheiner 2001). In all cases, the assumption of equal covariance between treatment levels was not violated.

## RESULTS

### *Sediment Selection by Scallops*

At the end of the experiment, the number of scallops on each sediment type in the absence of a predator was significantly different from the expected distribution (10 scallops per sediment type) (Table 1; Fig. 1). The number of scallops was significantly lower than expected on glass (Table 1). When a rock crab was present, the number of scallops was also significantly lower than expected on glass (Table 1; Fig. 1). Scallops distribution was not different than expected when a sea star was present (Table 1; Fig. 1).

Scallop distribution on the different sediment types over the duration of the experiment was influenced by predator presence and time (Table 2; Fig. 2). When alone, the number of scallops was highest on granule and pebble and lowest on glass (Fig. 2). Scallops selected all sediment types over glass when crabs were present (Fig. 2), but did not select a specific sediment type when sea stars were present. Scallops distribution changed from the initial distribution to selected sediments after 60 min when alone and after 180 min when a crab was present, and remained fairly stable for the rest of the trial. In contrast, scallop distribution when a sea star was present did not stabilize over the duration of the trial (Fig. 2). MANOVA generated four eigenvectors (i.e., squared canonical correlation) that explained 65.6% and 56.5% of the total variation in the model, for the predator presence and time effect, respectively. Of these, the first eigenvector explained 54.4% and 48.1% of the variation for predator presence and time effect, respectively. We interpreted the coefficients of the standardized canonical variates associated with these first eigenvectors (Scheiner 2001) (Table 2). For the predator effect, the number of scallops on granule and pebble contributed most to the significant MANOVA result, followed by sand and glass (Table 2). All coefficients had the same sign, indicating that the various sediments contributed in a similar direction to the predator effect (Table 2). For the time effect, sand and granule contributed most to the significant result. All coefficients had the same sign, and so the various sediments contributed in a similar direction to time effect.

TABLE 1.

Results of one-sample Hotelling  $T^2$  tests for the number of scallops (*Placopecten magellanicus*) on each sediment type at the end of the experiment (720 min) and the total proportion of time sea stars (*Asterias vulgaris*) and rock crabs (*Cancer irroratus*) spent on each sediment type compared to the expected distributions. Expected distribution for scallops = 10 scallops on each of glass, sand, granule, pebble; sea stars = 0.126 on each of glass, sand, granule, pebble, and 0.494 on "other"; rock crabs = 0.25 on each of glass, sand, granule, pebble. For scallop distribution when alone, linear dependency in the data matrix was corrected by removing data on granule. Data on granule and on pebble were removed for the sea star and rock crab analyses, respectively.

Analysis	Treatment	$T^2$	$F_{df1, df2}$	$p$	Mean $\pm$ 95% CI
Scallop distribution	Alone	75.51	17.97 <sub>3,5</sub>	<b>0.004</b>	Glass: $-2.095 \leq 2.875 \leq 7.845^*$ Sand: $6.263 \leq 9.375 \leq 12.49$ Pebble: $9.819 \leq 14.50 \leq 19.18$
	With sea star	28.02	4.003 <sub>3,4</sub>	0.104	
	With rock crab	103.1	14.72 <sub>4,4</sub>	<b>0.012</b>	Glass: $-2.945 \leq 3.500 \leq 9.945^*$ Sand: $1.544 \leq 12.25 \leq 22.96$ Granule: $-2.469 \leq 12.37 \leq 27.22$ Pebble: $1.678 \leq 11.63 \leq 21.57$
Sea star distribution	Alone	144.7	20.67 <sub>4,4</sub>	<b>0.006</b>	Glass: $0 \leq 0.020 \leq 0.091^*$ Sand: $0 \leq 0.047 \leq 0.157$ Pebble: $0 \leq 0.062 \leq 0.376$ Other: $0.282 \leq 0.804 \leq 1.00$
	With scallops	352.5	50.34 <sub>4,4</sub>	<b>0.001</b>	Glass: $0 \leq 0.016 \leq 0.075^*$ Sand: $0 \leq 0.114 \leq 0.555$ Pebble: $0 \leq 0.361 \leq 1.00$ Other: $0 \leq 0.381 \leq 1.00$
Rock crab distribution	Alone	243.2	57.90 <sub>3,5</sub>	<b>&lt;0.001</b>	Glass: $0 \leq 0.049 \leq 0.117^*$ Sand: $0.536 \leq 0.826 \leq 1.00^*$ Granule: $0 \leq 0.058 \leq 0.166^*$
	With scallops	388.4	92.48 <sub>3,5</sub>	<b>&lt;0.001</b>	Glass: $0 \leq 0.043 \leq 0.114^*$ Sand: $0.503 \leq 0.829 \leq 1.00^*$ Granule: $0 \leq 0.086 \leq 0.249^*$

df1 = numerator df; df2 = denominator df; CI = confidence interval.

Significant results are indicated in bold, and sediment types that contributed to the significant result (based on CI calculations) are indicated with an asterisk.

#### Sediment Selection by Sea Stars

Sea stars spent significantly less time than expected on glass when alone and when scallops were present (Table 1; Fig. 3).

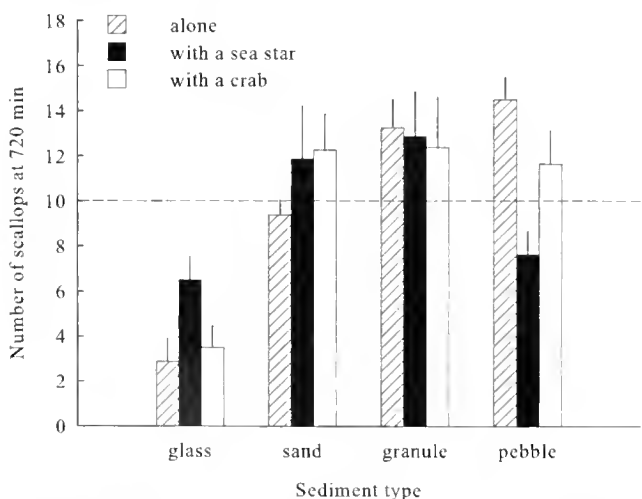


Figure 1. Mean number of juvenile sea scallops (*Placopecten magellanicus*) on each sediment type at the end of the experiment (720 min) when alone and in the presence of a sea star (*Asterias vulgaris*) and a rock crab (*Cancer irroratus*). Dashed line represents the expected distribution of 10 scallops per sediment type. Error bars are 1 SE,  $n = 8$ .

When sea stars alone and sea stars in the presence of scallops were directly compared, the distribution did not change significantly (Table 3). In both situations, sea stars spent a large proportion of time on "other" substrate. However, sea stars spent less time on vertical surfaces in the presence of scallops than when alone ( $t_{14} = 2.83$ ,  $P = 0.013$ ). Sea star predation rate on scallops on the 4 different sediments did not differ from the expected rate (Table 4, Fig. 4).

#### Sediment Selection by Rock Crabs

Rock crabs spent significantly more time than expected on sand, and so significantly less time than expected on the other sediment types (Table 1; Fig. 3). When on sand, crabs spent large proportions of the total observation time (~80%) wholly or partially buried in the sand with little activity. When crabs alone and crabs in the presence of scallops were directly compared, the distribution did not change (Table 3). Rock crab predation rate on scallops on the 4 different sediments did not differ from the expected rate (Table 4; Fig. 4).

## DISCUSSION

#### Sediment Selection by Animals

Our study showed that juvenile sea scallops (*Placopecten magellanicus*, ~30 mm SH) selected specific sediment types. In the absence of a predator, scallops selected granule and pebble bottom

TABLE 2.

Split-plot MANOVA results for distribution of scallops (*Placopecten magellanicus*) over time in the presence and absence of a predator (*Asterias vulgaris*, *Cancer irroratus*).

Source of Variation	Error Term	$F_{0.01,df2}$	$p$	Glass	Sand	Granule	Pebble
P	M (P)	2.32 <sub>8,38</sub>	<b>0.039</b>	-0.962	-1.267	-1.458	-1.464
T	T $\times$ M (P)	3.54 <sub>48,1008</sub>	<b>&lt;0.001</b>	0.887	1.134	1.130	0.945
P $\times$ T	T $\times$ M (P)	1.23 <sub>96,1008</sub>	0.075				

P = predator presence, T = time, M = tank, F = F-ratio for Pillai trace. Significant results are indicated in bold. Coefficients of the first standardized canonical variate are provided to aid interpretation of significant main effects. Similar signs of coefficients indicate a positive correlation between sediment types, and magnitude of values indicates the relative contribution of each sediment type to the significant result.

over the other sediment types. Similar results were observed by Barbeau (unpublished data) when smaller juvenile scallops (9.0–10.4 mm SH) were offered glass bottom, sand, granule, and a pebble/cobble mixture simultaneously. Also, using pair-wise

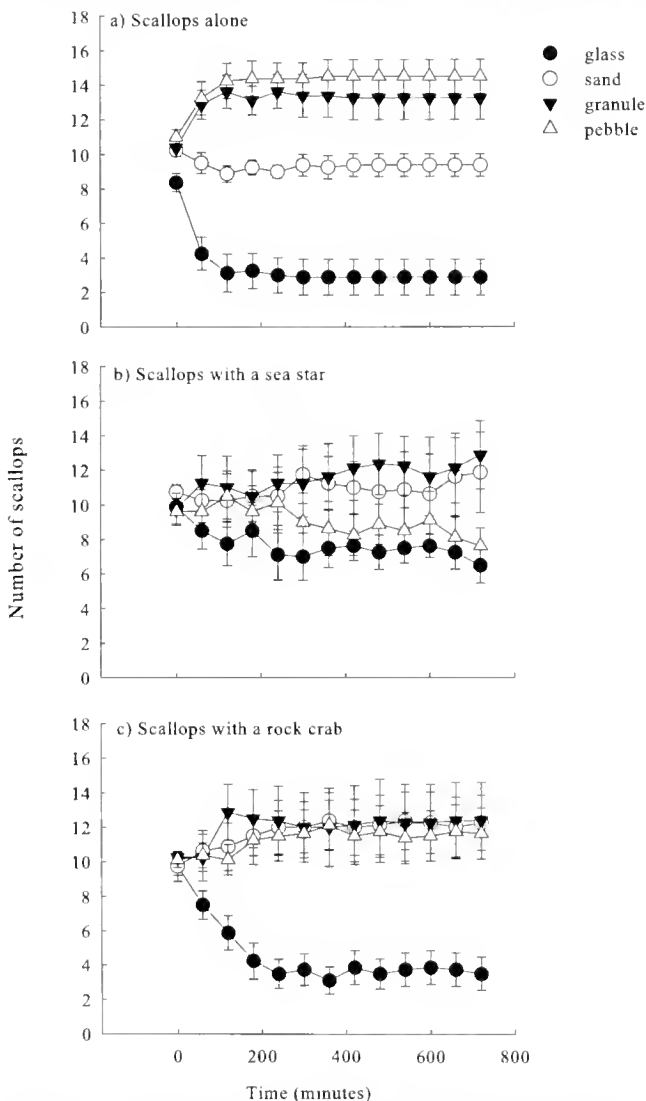


Figure 2. Number of juvenile sea scallops (*Placopecten magellanicus*) observed on each sediment type at 1-h intervals, when (a) alone, (b) with a sea star (*Asterias vulgaris*) and (c) with a rock crab (*Cancer irroratus*). Observations began after a 5 min acclimation period. Mean  $\pm$  SE, n = 8.

choice experiments, Bourgeois (2004) found that juvenile scallops (~25 mm SH) selected granule and a coarse sand/shell sediment over homogeneous sand. In our current experiment, sediment selection of scallops changed in the presence of sea stars (*Asterias vulgaris*). Scallops no longer avoided glass bottom; instead, scallops did not select any specific sediment type over others, and their distribution did not differ from the expected distribution of 10 scallops per sediment type. This probably resulted because juvenile sea scallops usually swim when encountered by sea stars (Barbeau & Scheibling 1994, Wong & Barbeau 2003), and so

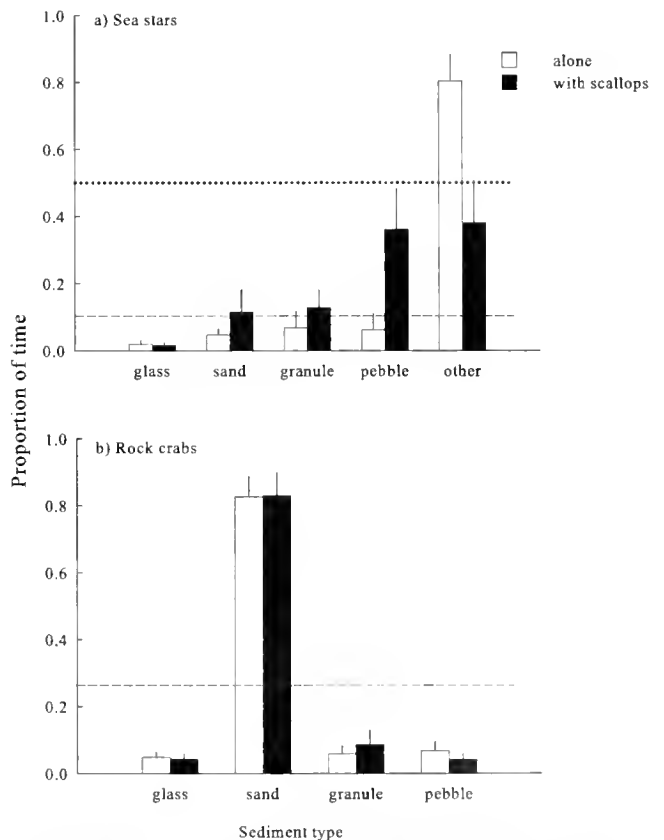


Figure 3. Mean proportion of time (a) sea stars (*Asterias vulgaris*) and (b) rock crabs (*Cancer irroratus*) spent on each sediment type over the duration of the experiment, when alone and when juvenile sea scallops (*Placopecten magellanicus*) were present. Dotted line represents expected value on "other" substrate; dashed line represents expected value on glass, sand, granule and pebble sediments. Error bars are  $\pm$  SE, n = 8.

TABLE 3.

Results for two-sample Hotelling  $T^2$  tests for the distribution of sea stars (*Asterias vulgaris*) and rock crabs (*Cancer irroratus*) in the absence and presence of scallops (*Placopecten magellanicus*). Data on granule and on pebble were removed for sea star and rock crab analyses, respectively.  $df_1$  = numerator df,  $df_2$  = denominator df.

Analysis	$T^2$	$F_{df_1, df_2}$	$P$
Sea star	12.28	2.41 <sub>1, 11</sub>	0.112
Rock Crab	0.016	0.005 <sub>3, 12</sub>	0.997

redistributed relatively frequently. Contrary to these results, sediment selection by scallops when rock crabs (*Cancer irroratus*) were present did not change from when scallops were alone, because scallops still avoided glass. Interestingly, scallops did not avoid sand when a crab was present, even though crabs clearly selected sand over other sediment types. Although crabs spent the majority of their time on sand buried, crabs did consume some scallops on this sediment. Previous experiments showed that rock crabs spend only a small percent of their time foraging (<25%), but that when they do forage, they are very effective at encountering, capturing and consuming scallops (Barbeau & Scheibling 1994, Wong & Barbeau 2003, Wong & Barbeau 2005). Scallops may not have avoided sand sediment when crabs were present because juvenile scallops tend to tightly close their valves upon encounter with crabs (which provides some protection against predation), instead of the typical swimming escape response used to evade sea stars (Barbeau & Scheibling 1994). Thus, the distribution of scallops in the presence of predators seems to be dependent on the type of antipredator strategy used by the prey.

In general, the distribution of predators did not depend on the presence or absence of scallops, because distributions did not change significantly between the two situations. Sea stars avoided glass bottom and rock crabs spent more time than expected on sand regardless of whether scallops were present or not. Sea stars did, however, spend less time on vertical surfaces when scallops were present than when they were absent; clearly, the presence of scallops induced sea stars to move to the sediments and forage. Because the selection for specific sediment types by predators was generally not influenced by the presence or absence of scallops, sediment choice by scallops was more likely to be dependent on the location of predators than vice-versa.

Our results are consistent with field observations of scallops, sea stars and rock crabs on specific sediment types. In the field,

TABLE 4.

Results for one-sample Hotelling  $T^2$  tests for predation rate (number of scallops consumed over 12 h) of sea stars (*Asterias vulgaris*) and rock crabs (*Cancer irroratus*) on each sediment type, compared to the expected number of scallops consumed (sea stars: glass = 0.230, sand = 0.420, pebble = 0.270; rock crabs: glass = 0.374, sand = 1.323, granule = 1.323, pebble = 1.243). For sea stars, data on granule were removed to correct for the linear dependency in the data.  $df_1$  = numerator df,  $df_2$  = denominator df.

Analysis	$T^2$	$F_{df_1, df_2}$	$P$
Sea star	3.000	0.71 <sub>3, 5</sub>	0.584
Rock crab	9.135	1.31 <sub>4, 4</sub>	0.401

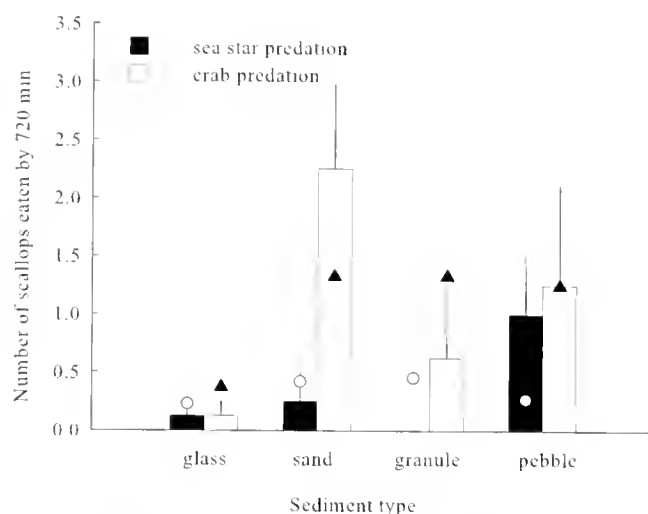


Figure 4. Mean number of juvenile sea scallops (*Placopecten magellanicus*) consumed by sea stars (*Asterias vulgaris*) and rock crabs (*Cancer irroratus*) by the end of the experiment. Open circles and closed triangles represent expected number of scallops consumed for sea stars and rock crabs, respectively, on each sediment type (calculated using the observed distributions of scallops at 720 min; Fig. 1). Sea stars did not consume any scallops on granule. Error bars are 1 SE,  $n = 8$ .

juvenile scallops (<50 mm SH) as well as adult scallops tend to be found on gravelly- or mixed sediments (Thouzeau et al. 1991, Barbeau et al. 1996, Wong et al. 2005), which we observed in our laboratory experiment. Juvenile scallops often move to gravelly sediments after the initial postlarval phase (spat) settle on various materials such as hydrozoans, amphipod tubes (Larsen & Lee 1978), shells of live *P. magellanicus* (Naidu 1970), shell fragments (Caddy 1968), bryozoans (Baird 1953, Caddy 1972) and red algae (Naidu 1970). By choosing sediments that provide vertical relief, spat may attain protection from shifting bottom sediments or from predation (Larsen & Lee 1978). Scallops species other than *P. magellanicus* also select specific sediment types when juveniles. For example, juvenile bay scallops (*Argopecten irradians*) attach to sea grass blades to reduce the probability of detection by predators (Pohle et al. 1991).

Our results for sea stars are also mostly consistent with field observations. Although found on most sediment types in the field, sea stars are usually observed on coarse sediments such as granule or cobble (Langton & Uzmann 1989). However, Himmelman & Dutil (1991) also observed high densities of sea stars (*A. vulgaris* and *Leptasterias polaris*) on bedrock. The selection of the vertical surface of the tank, especially when scallops were absent, has been observed during previous laboratory experiments (M. Wong, pers. obs.), and may be an experimental artifact. The burial behavior of rock crabs in sandy sediments has been observed in the field (Scarrott & Lowe 1978), and is well documented for other crab species, such as the dungeness crab (*Cancer magister*) and red rock crab (*Cancer productus*) (Bellwood 2002, McGaw 2005).

#### Sediment Selection and Predator-prey Interactions

In our study, we observed sea stars and crabs consuming scallops on all sediment types (except granule for sea stars). These results are comparable to those for similar-sized juvenile scallops (24–28 mm SH) in Wong and Barbeau (2003), where sediment type did not affect sea star and rock crab predation rate. However,

in Wong and Barbeau (2003), predation rate of smaller scallops (11–15 mm SH) by sea stars decreased with increasing particle size of sediments. If we had used such smaller scallops in our current study, we may also have detected an effect of sediment particle size on predation rate.

The effect of sediment type on predation of sea scallops by sea star and crab predators has been examined in field experiments in Baie des Chaleurs, Gulf of St. Lawrence, Canada (Stokesbury & Himmelman 1995). In these experiments, tethered scallops were used to quantify predation of juvenile sea scallops (35–45 mm SH) at sites with sand, gravel (composed of pebble and small cobble), or bedrock. Predator-related mortality of scallops was significantly higher at a site with sand than a site with gravel or bedrock. Furthermore, scallop shell remains indicated that ~50% to 80% of scallop predation was by decapods (*Cancer irroratus*, *Homarus americanus*, *Hyas* spp.), whereas the remaining predation was by sea stars (*Asterias vulgaris*, *Crossaster paposus*, *Leptasterias polaris*). Because density of rock crabs was positively correlated with predator-related mortality of tethered scallops, and the highest density of these crabs ( $0.56 \pm 0.43$  individuals per  $10 \text{ m}^2$ ; mean  $\pm$  SD) was at the sandy site, these results may be directly related to predator density and not to differential foraging abilities of predators on the various sediment types.

The complexity of sediment types, and not simply the size of sediment particles, can also influence predator-prey interactions. Talman et al. (2004) found that mortality of tethered New Zealand scallops (*Pecten novaezelandiae*), caused by predation, decreased as habitat complexity increased. In this case, habitat complexity was defined in terms of biological features (e.g., diversity of biota) and physical features (e.g., number of sediment types, density, or structure). In our experiments, granule and pebble could be considered heterogeneous sediments because they provide physical crevices, whereas glass and sand are homogeneous sediments. However, we did not observe decreased predation on more complex sediment types, as observed for the New Zealand scallops. This may have resulted from the absence of biotic heterogeneity in our sediment types.

The lack of effect of sediment type on predation in our current study may be directly associated with the type of antipredator behavior used by sea scallops. As discussed in Wong and Barbeau (2003), there are two types of behaviors that prey can use to reduce predation risk: avoidance behaviors and escape behaviors (Sih 1987). Prey that use avoidance behaviors reduce the probability of detection and thus the probability of being encountered by predators, so these prey are likely to make use of features of the substrate to evade predators. However, prey that use escape behaviors after an encounter with a predator reduce the probability of being attacked or captured, and would be less likely to use substrate features. Indeed, juvenile sea scallops (*P. magellanicus*,  $\geq 11$  mm SH) use active escape mechanisms by swimming horizontally through the water column, and do not actively seek refuge by hiding in crevices between sediment particles (as do Iceland scallops, *Chlamys islandica*; Arsenault & Himmelman 1996) or by attaching to sea grass blades (as do juvenile bay scallops; *Argopecten irradians*, Pohle et al. 1991). The lack of effect of sediment type may also be related to predator foraging behaviors on the different sediments. Specific behaviors, such as searching for prey, handling prey and encounters between predators and prey would require quantification to further investigate this (as done in Wong & Barbeau 2003).

#### Implications for Future Experiments and Aquaculture

The experimental design used in our study allowed comprehensive examination of sediment selection by juvenile sea scallops, sea stars and rock crabs. Observed distributions of animals were compared with expected distributions. Further, distributions when animals were alone were compared with distributions when they were in the presence of a predator or prey. While we were able to identify selection of sediment types, our experimental design did not evaluate preference for sediment types. Preference is an active behavioral choice, and can only be identified if it is not confounded with "accessibility" of that choice (Underwood et al. 2004). Accessibility in our case refers to the ease with which a sediment type can be found or occupied (Olabarria et al. 2002). For example, scallops may be found mostly on a pebble sediment because they have difficulty swimming away from the cracks and crevices between sediment particles, and not because they actively chose this particular sediment type over others. The experimental design necessary to identify preference would include treatments that allow animals to choose between sediment types, as well as treatments that do not allow a choice of sediments (Olabarria et al. 2002, Barbeau et al. 2004). In our experiments, additional tanks where each divided segment is filled with the same sediment type would need to be included (i.e., replicated treatments would include only glass, sand, granule or pebble sediment). Preference would be evident if animals chose a particular sediment more often in the choice situation (i.e., in choice treatments) than expected by chance when no choice is available (i.e., in no-choice treatments). Thus, the observed association of animals on particular sediment types in our experiments indicates selection of that sediment, but not necessarily an active behavioral choice.

Our results have implications for the bottom culture of scallops, and suggest that aquaculturists should carefully consider the sea bottom characteristics of the site before seeding scallops. Juvenile scallops select more heterogeneous sediment types (e.g., granule or pebble) over more homogeneous substrates (sand or flat, hard bottoms). Therefore, dispersal of scallops away from a site will be higher on less suitable sediment types. This has been observed in early seeding trials off the Magdalen Islands, Quebec, Canada, where sea scallops were released on sand (Picard & Vigneau 1992). Dispersal from certain sediment types has been observed in other scallop species, such as bay scallops (*Argopecten irradians*), which swim from an unnatural sediment (sand) to their normal substrate (eelgrass blades) (Winter & Hamilton 1985), and great scallops (*Pecten maximus*), which have a higher probability of swimming from bedrock than from sediments suitable for recessing (Baird 1958, Baird 1966). Furthermore, in our system, sea scallop dispersal will be higher if sea stars are present, because sea star encounter seems to be a primary trigger for scallop swimming. Encounter with crab predators may also trigger swimming of sea scallops, but not to the extent of encounters with sea stars. Note, however, that crabs can be much more effective scallop predators than sea stars (Barbeau & Scheibling 1994). Based on research with another scallop species (Talman et al. 2004), the foraging efficiency of both predator types may be reduced in habitats with high structural complexity (e.g., high density of macrophytes). Additionally, the sediment type of an aquaculture site should be considered in terms of scallop growth. Brand (1991) reported faster growth of various scallop species (e.g., *P. magellanicus*, *A. irradians*, *P. maximus*) on sediments with little silt or mud. Thus, seeding sea scallops onto sediments with large particle sizes may lead to faster growth. Generally, sediment type remains an impor-

tant factor that can influence the success of bottom culture operations by affecting predator-prey interactions, scallop movement, and scallop growth.

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## EFFECT OF MICROALGAL CONCENTRATION AND WATER TEMPERATURE ON THE PHYSIOLOGY OF THE CARIBBEAN SCALLOPS *ARGOPECTEN NUCLEUS* AND *NODIPECTEN NODOSUS*

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**ABSTRACT** *Argopecten nucleus* and *Nodipecten nodosus* are two Caribbean scallops occurring in Colombia, which have recently been selected for artificial culture based on their high commercial value. As part of an effort to develop culture technology for these species, we studied the effects of food concentration and temperature on feeding rates (filtration, ingestion, and absorption), oxygen consumption, ammonium excretion and scope for growth in adults of the two scallop species. We tested the effects of four concentrations of the microalga *Isochrysis galbana* (10, 20, 40 and 60 cells  $\mu\text{L}^{-1}$ ) at three water temperatures (20, 25 and 28°C), at a constant salinity of 36‰. The results showed that increases in food concentration induced increases in feeding rate, oxygen consumption and growth potential, but at values of 60 cells  $\mu\text{L}^{-1}$  these variables decreased, indicating saturation of the digestive tract. The excretion rate increased at low food concentrations, particularly at the middle (25°C) and/or at the highest temperature tested (28°C). This suggested utilization of endogenous proteins as a supplementary energy source under these conditions. The increase in temperature had no significant effect on the feeding variables or on the scope for growth of *A. nucleus*, but raised the *N. nodosus* ones. All the physiological rates for the two species were similar except for oxygen consumption, which was greater in *N. nodosus* than in *A. nucleus*. Using values obtained for the algae concentrations, which produced the greatest growth potential, the optimal value for *I. galbana* for both the scallops was 40 cells  $\mu\text{L}^{-1}$ , whereas the optimal temperature for *N. nodosus* was 25°C. There was no single optimal temperature for *A. nucleus*, which functioned equally well at between 20°C and 28°C.

**KEY WORDS:** scallop physiology, scallop culture, *Argopecten nucleus*, *Nodipecten nodosus*, Colombia, Caribbean.

### INTRODUCTION

Temperature and concentration of particulate food are two of the main factors that affect bivalve filter feeders in relation to their growth (Wilson 1987), survival (Paul 1980), gonadal conditioning (Martínez et al. 2000, Martínez & Pérez, 2003) and physiology (Bricelj, et al. 1987, Navarro & Iglesias 1995, Navarro et al. 2000). Studies carried out on the physiology of bivalve filter feeders in response to broad ranges of microalgal concentrations (Griffiths & King 1979, Bacon et al. 1998, MacDonald et al. 1998, Velasco & Navarro 2002, 2003) and/or water temperature (Alí 1970, Winter 1970, Bougrier et al. 1995, Sicard et al. 1999, Laing 2000) have shown that the filtration rates, ingestion, absorption, oxygen consumption and/or excretion become modified with changes in these parameters, and allow maintenance of high values in scope for growth within given ranges of the parameters depending on the species observed.

*Argopecten nucleus* (Born, 1780) and *Nodipecten nodosus* (Linné, 1758) are two scallops of commercial importance from the Caribbean coast of Colombia. These species are epibenthic filter feeders and may coexist on sandy bottoms from 10–100 m depth. *A. nucleus* is a moderately-sized, unattached species (length = 50 mm), whereas *N. nodosus* is a larger (length = 150 mm) species, which remains attached to hard substrates. No naturally occurring beds of these scallops have been encountered in the sea off Colombia; population aggregates have been maintained in artificial cultures, produced from the capture of (scarce) naturally occurring seed obtained in spat collectors (maximal of 6–77 spats collector<sup>-1</sup>) (Urban, 1999). These two scallop species have shown high growth rates in suspended cultures, reaching commercial sizes within 11 mo (Urban 1999, INVEMAR 2003). It is known that growth and survival of *N. nodosus* is negatively affected by ex-

treme temperatures (<20°C and >29°C), low salinities (<29‰) and high turbidity (Rupp & Parsons 2004, Rupp et al. 2005).

Because the scarce availability of wild “seed” does not support commercial cultures of these scallops, it was of interest to develop the technology for seed production under hatchery conditions. Success in seed production depends on the identification of optimal environmental parameters for maintenance of the adults, larvae and postlarvae in the laboratory. Because the two species under study occur naturally in relatively high and constant salinity, whereas the temperature and environmental food offerings may vary considerably, we selected the latter two variables of interest for the present study. In preliminary studies we determined that the chrysophyte *Isochrysis galbana* produced the best scope for growth in the two scallop species (Velasco, in press). This study was carried out to determine the effect of different concentrations of *I. galbana* and different temperatures on physiological variables related to feeding (filtration rates, ingestion and absorption), oxygen consumption, excretion and scope for growth of *Argopecten nucleus* and *Nodipecten nodosus* adults.

### MATERIALS AND METHODS

#### Collection and Maintenance of Scallops

Specimens of *Argopecten nucleus* (mean length  $47 \pm 5.3$  mm and dry weight  $1.25 \pm 0.35$  g) and *Nodipecten nodosus* (mean length  $71 \pm 9.5$  mm and dry weight  $2.26 \pm 0.85$  g) were collected at the Bahía Neguanje bivalve culture station (Lat. 11° 20' N., Long. 74.05° W.), in Santa Marta, Colombia. The water temperature in this area ranges between about 22°C and 30°C, with salinities between 33 and 37‰ and environmental seston concentrations between 0.2 and 4.7 mg L<sup>-1</sup>; the organic content of the seston ranged between 15% and 60% (Urban 1999).

The scallops were transported wet, at a temperature similar to that of the environment, to the Mollusc and Microalgae Laboratory

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of the Universidad del Magdalena at Taganga (Lat. 11°16'N, Long. 74°11'W), where they were cleaned of epibionts, and individually marked. Acclimation to laboratory tanks was achieved by maintaining the scallops for one week in static seawater in a 250 L tank at a salinity of 36 ‰ and temperature of 25°C. The water was aerated with a compressed air bubbler, and a daily food ration of *Isochrysis galbana* equivalent to 3% of the dry weight of the scallops. The water was renewed every 24 h.

### Experimental Design

A factorial design was used in which physiological parameters of the scallops were measured at three different water temperatures (20, 25°C, and 28°C) and four different feeding regimes with *Isochrysis galbana* at 2, 4, 8 and 12 mg L<sup>-1</sup> (10, 20, 40 and 60 cells µL<sup>-1</sup> respectively). Each of these 12 treatments was applied to seven individual scallops of each species selected haphazardly from the original groups of specimens obtained. The salinity was maintained at 36‰ in all the experiments; complete oxygen saturation was maintained in the water over a period of 15 h, of which 12 h represented an acclimation period in the test system with a three hour period for taking the measurements.

The experimental diets were administered using a flow-through system consisting of 16 chambers (0.8 L for *A. nucleus* and 1.6 L for *N. nodosus*), designed following Riisgård (1977). A constant flow (150 ± 10 mL min<sup>-1</sup>) of the experimental diet was directed by gravity from a mixing tank into each chamber; 14 chambers were used for individual bivalves (7 individuals of each species) and 2 chambers contained empty valves, which served as controls. Normal valve opening by test specimens was monitored and individuals that failed to open normally were eliminated from the experiment.

The experimental diets were prepared by mixing appropriate volumes of 1-µm microfiltered seawater and the microalgae *Isochrysis galbana*, which had been cultured in F/2 medium (Guillard 1974), after direct cell counts of cell densities by hemacytometer.

### Characterization of Feeding Ration

Each experimental diet was analyzed in terms of total particulate matter (TPM) and particulate organic matter (POM). Water was sampled from the outflow of both control chambers every hour. Each sample was divided into three subsamples, which were filtered onto tared Millipore glass fiber filters (α = 0.45 µm), which had been prewashed in distilled water and preashed at 450°C for 4 h. Filters with samples were rinsed with isotonic ammonium formate to remove salt and prevent cell lysis, and were dried at 70°C for 48 h to determine the TPM (mg L<sup>-1</sup>). POM (mg L<sup>-1</sup>) was determined as the loss in weight of the samples after ashing at 450°C for 4 h.

### Determination of Physiological Feeding Variables

These variables were determined by the method of biodeposition described by Iglesias et al. (1998), as validated by Navarro and Velasco (2003) using the flow-through chamber method described by Riisgård (2001). Feces and pseudofeces were quantitatively collected every 3 h using Pasteur pipettes. The mass and organic content of the material deposited by each test bivalve were estimated separately using the method described above for the diet analysis. The equations for the physiological variables were the same as those of Iglesias et al. (1998).

### Filtration Rate

Filtration rate (FR) represents the amount of particulate material removed from the water per unit time.

$$\text{Inorganic filtration rate} \quad \text{IFR (mg h}^{-1}\text{)} = \text{IRR} + \text{IER} \quad (1)$$

$$\text{Organic filtration rate} \quad \text{OFR (mg h}^{-1}\text{)} = \text{IFR} * (\text{POM/PIM}) \quad (2)$$

$$\text{Total filtration rate} \quad \text{FR (mg h}^{-1}\text{)} = \text{IFR} + \text{OFR} \quad (3)$$

where: *IFR* = inorganic filtration rate (mg h<sup>-1</sup>); *IRR* = rate of production of pseudofeces inorganic matter (mg h<sup>-1</sup>), *IER* = rate of production of feces inorganic matter (mg h<sup>-1</sup>), *POM* = particulate organic matter (mg L<sup>-1</sup>) and *PIM* = particulate inorganic matter (mg L<sup>-1</sup>).

### Ingestion Rate

When no pseudofeces is produced the total filtration and organic matter filtration rates are represented, respectively, by the total (IR) ingestion rate and organic ingestion rates (OIR). When pseudofeces is produced, the IR is calculated by subtraction of the rate of pseudofeces production from the FR (Bayne et al. 1993).

$$\text{IR (mg h}^{-1}\text{)} = \text{FR} - \text{RR} \quad \text{OIR (mg h}^{-1}\text{)} = \text{OFR} - \text{ORR} \quad (4)$$

where: *RR* = rate of production of total pseudofeces (mg h<sup>-1</sup>) and *ORR* = rate of production of pseudofeces organic matter (mg h<sup>-1</sup>).

Efficiency (AE) and rate of absorption (AR): represent material ingested, which is absorbed per unit time.

$$\text{AE}(\%) = \text{AR}/(\text{OFR} - \text{ORR}) * 100 \quad (5)$$

$$\text{AR (mg h}^{-1}\text{)} = \text{OIR} - \text{OER} \quad (6)$$

where: *OER* = rate of production of feces organic matter (mg h<sup>-1</sup>).

Oxygen consumption (CO: mL O<sub>2</sub> h<sup>-1</sup>) and ammonia excretion (UR: µg NH<sub>3</sub>-N h<sup>-1</sup>): were determined on fed animals by placing them in individual chambers (0.8 and 3 L) after rinsing the chambers with 25% HCl and filling with <1-µm filtered seawater. Chambers were sealed and incubated for 2 h at the same temperature in which they have been fed, alongside a control chamber devoid of specimens. Two water samples were then taken from each experimental chamber to determine oxygen consumption rates and rates of excretion. Oxygen consumption was never measured at ambient oxygen tension lower than 70% saturation. Oxygen concentration was estimated by the Winkler method, modified after Carritt and Carpenter (Strickland & Parsons 1972) and ammonia excretion was determined by the phenol-hypochlorite method (Solorzano 1969).

Scope for growth (SFG): is a physiological index of energy balance to estimate production (growth + reproduction) by an individual animal. It was calculated from the equation given by Widdows (1985) after converting all the physiological rates to energy equivalents (J h<sup>-1</sup>):

$$\text{SFG} = A - (R + U) \quad (7)$$

where: *A* = Energy absorbed (J h<sup>-1</sup>) = *AR* mg h<sup>-1</sup> × 11.40 J mg<sup>-1</sup> (Velasco, in press), *R* = Oxygen consumption (J h<sup>-1</sup>) = *OC* mL O<sub>2</sub> h<sup>-1</sup> × 20.08 J (Gnaiger 1983), *U* = Ammonia excretion (J h<sup>-1</sup>) = *UR* mg NH<sub>3</sub>-N h<sup>-1</sup> × 24.8 J (Elliot & Davison 1975).

### Standardization of Variables

The physiological rates were converted to a standard individual of 1-g dry tissue weight and with a macroscopic gonadal stage of I; for this, the soft tissues were dried at 70°C for 48 h, and then individually weighed. Standardization used the equation of Bayne et al. (1987).

$$Y_{ts} = (1 \text{ g}/W_e)^{b1} \times Y_e \quad (8)$$

$$Y_{ms} = (1 \text{ E})^{b2} \times Y_{ts} \quad (9)$$

where  $Y_{ts}$  = physiological rate of an individual of standard size (1 g),  $Y_e$  = uncorrected rate,  $W_e$  = weight of the experimental individual,  $b1$  = dependence of the physiological rates on the sizes of the individuals,  $Y_{ms}$  = physiological rate of the individual at a standard stage of maturity (I),  $E$  = stage of maturity of the experimental individual (between I and 4) and  $b2$  = dependence of the physiological rates on the stage of maturity of the individuals. The "b" values used for each physiological rate were determined from exponential regressions between the physiological measurements, the dry weights, and stage of maturity of specimens of different sizes and degrees of maturity of the two scallop species. For the rate of production of biodeposits, the  $b1$  values were 0.98 and 0.41 and for  $b2$  they were -0.67 and -0.44 for *Argopecten nucleus* and *Nodipecten nodosus*, respectively. For the oxygen consumption rates, the  $b1$  values were 1.52 and 0.43 and for  $b2$  they were 0.32 and 0.52 for *A. nucleus* and *N. nodosus*, respectively. Finally, for the rates of excretion, the  $b1$  values were 1.16 and 0.97 for *A. nucleus* and *N. nodosus*, respectively, and no standardization was carried out related to the stage of maturity, because this factor did not affect the excretion rate.

### Statistical Analyses

Tests were carried out on the normality and the homogeneity of the variances of all the physiological variables, and transformed to comply with these requisites. Clearance, filtration, ingestion, absorption and SFG were transformed to square, oxygen consumption was transformed to square roots, and a rank transformation was applied to the absorption efficiency and excretion rate data. Factorial analyses of variance were carried out to establish the degree of influence of the food concentration and temperature, and the interactions of these two factors on each of the physiological variables. One-way analyses of variance were carried out to make specific comparisons between treatments, and to determine significant differences among them using a Bonferroni multiple range test. Correlation analyses were carried out to establish associations among the physiological variables. Finally, analyses of covariance were carried out to compare the physiological variables between the two species. All the statistical analyses were carried out using Statgraphics-plus 5.0 software, with an  $\alpha$  of 0.05 for all tests of significance.

## RESULTS

### Feeding Rates

The highest rates of filtration, ingestion and absorption by *Argopecten nucleus* were 85.2, 55.5 and 52.4 mg h<sup>-1</sup>, respectively, whereas the lowest rates, respectively, were 7.1, 4.6 and 4.1 mg h<sup>-1</sup>. In *Nodipecten nodosus* the filtration rates varied between 5.8 and 83.5 mg h<sup>-1</sup>; ingestion rates between 3.7 and 56.7 mg h<sup>-1</sup> and the absorption rates between 3.2 and 53.3 mg h<sup>-1</sup> (Fig. 1). The

food concentration significantly affected the rates of filtration, ingestion and absorption of *A. nucleus* ( $df = 3$ ,  $P < 0.001$ ) and *N. nodosus* ( $df = 3$ ,  $P < 0.0001$ ). Increase in physiological rates occurred with increases in the food concentration, with the highest values observed at 40 cells  $\mu\text{L}^{-1}$ . At food concentrations of 60 cells  $\mu\text{L}^{-1}$  all the rates decreased, except in *N. nodosus* at 20°C, where they continued to increase.

Temperature did not significantly affect the feeding rates of *Argopecten nucleus* ( $df = 2$ ,  $F = 1.13$ ,  $P = 0.3316$ ) whereas the contrary was true of *Nodipecten nodosus* ( $df = 2$ ,  $F = 22.22$ ,  $P = 0.0001$ ), although only when the scallops were presented with intermediate food concentrations (20 and 40 cells  $\mu\text{L}^{-1}$ ): at these food concentrations, the low temperature (20°C) and the high temperature (28°C) produced a significant decrease in the physiological variables related to feeding. The analysis of covariance showed that the feeding rates of both scallop species were statistically similar ( $df = 1$ ,  $P < 0.0828$ ). There was production of pseudofeces in all the treatments: in *N. nodosus* this varied between 14% and 52% of the material filtered, whereas in *A. nucleus* this range was between 18% and 47%, with the highest values observed in the treatments containing the highest food concentrations and at extremes of temperature.

### Absorption Efficiency

The absorption efficiency of *Argopecten nucleus* varied between 60.9% and 96.3%, whereas that of *Nodipecten nodosus* was between 74.5% and 97.5% (Fig. 2). The concentration of microalgae had a statistically significant influence on the absorption efficiency of both scallop species ( $df = 3$ ,  $P < 0.0020$ ), but a direct relation was not verified. The values obtained at 40 cells  $\mu\text{L}^{-1}$  were significantly higher than those at 20 cells  $\mu\text{L}^{-1}$ . Temperature affected the efficiency of absorption of *A. nucleus* ( $df = 2$ ,  $F = 6.33$ ,  $P = 0.0034$ ), with higher values obtained at 25°C than at the other temperatures. In *N. nodosus* the absorption efficiency was not significantly affected by the temperature ( $df = 2$ ,  $F = 2.83$ ,  $P = 0.0666$ ). The analysis of variance did not detect significant differences between the absorption efficiencies of the two species ( $df = 1$ ,  $F = 0.01$ ,  $P = 0.8289$ ).

### Oxygen Consumption Rate

The oxygen consumption of *Argopecten nucleus* varied between 0.18 and 1.05 mL O<sub>2</sub> h<sup>-1</sup> and that of *Nodipecten nodosus* was between 0.5 and 1.63 mL O<sub>2</sub> h<sup>-1</sup> (Fig. 3A). The factorial analysis showed that the oxygen consumption by the scallops was significantly affected by the food concentration ( $df = 3$ ,  $P < 0.0001$ ) and temperature ( $df = 2$ ,  $P < 0.0001$ ). In *A. nucleus* the oxygen consumption rates were significantly lower at the lowest algal concentration (10 cells  $\mu\text{L}^{-1}$ ), whereas in *N. nodosus* the highest values were obtained at 40 cells  $\mu\text{L}^{-1}$ . Increase in temperature increased oxygen consumption, with statistically higher values at 25°C and 28°C in *A. nucleus* and at 28°C in *N. nodosus*. The analysis of covariance showed that the oxygen consumption by *N. nodosus* was significantly higher than that of *A. nucleus* ( $df = 1$ ,  $F = 35.13$ ,  $P = 0.0001$ ).

### Excretion Rate

Ammonium production by *Argopecten nucleus* varied between 91.9 and 326.6  $\mu\text{g h}^{-1}$ , whereas that of *Nodipecten nodosus* ranged from 84.2–340.4  $\mu\text{g h}^{-1}$  (Fig. 3B). The factorial analysis of variance showed that the rate of excretion by the two scallop species

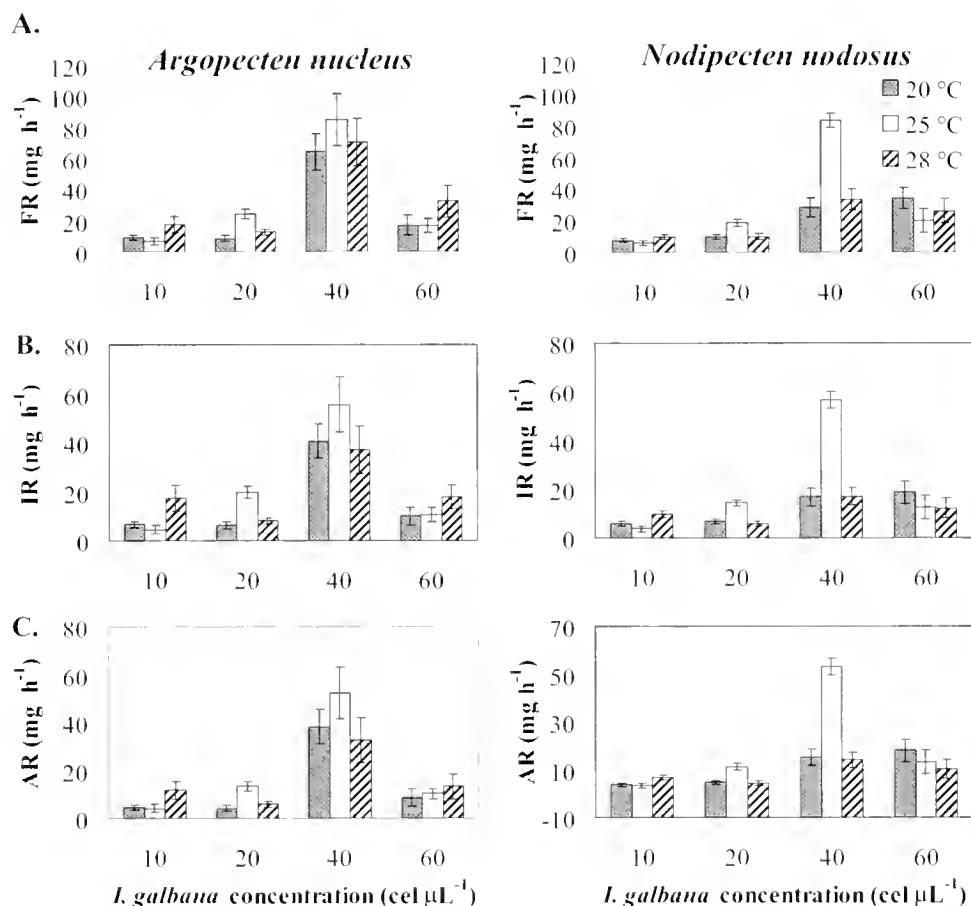


Figure 1. *Argopecten nucleus* and *Nodipecten nodosus*. Feeding rates under different concentrations of *Isochrysis galbana* and water temperatures. A. Filtration rates, B. ingestion rates and C. Absorption rates.

was significantly affected by the microalgal concentration ( $df = 3$ ,  $P < 0.0016$ ) and by the water temperature ( $df = 2$ ,  $P < 0.0030$ ). The excretion rates measured at the lowest food concentration (10 cells  $\mu\text{L}^{-1}$ ) were significantly higher than those obtained at higher food concentrations (40 cells  $\mu\text{L}^{-1}$ ). The temperature affected the excretion rate of these scallops only at low food concentrations (10 and 20 cells  $\mu\text{L}^{-1}$ ), and produced significantly higher values at 25 °C than at 20 °C ( $df = 2$ ,  $P < 0.0300$ ). At higher food concentrations, the excretion rate was not influenced by temperature ( $df = 2$ ,  $P > 0.1275$ ). No statistical differences were detected between the rates of ammonium release by the two scallop species ( $df = 1$ ,  $F = 0.08$ ,  $P = 0.7841$ ) using the analysis of covariance.

#### Scope for Growth

The scope for growth of *Argopecten nucleus* fluctuated between 33.2 and 580.2 J h<sup>-1</sup> and that of *Nodipecten nodosus* between 9.6 and 582.2 J h<sup>-1</sup> (Fig. 4). The correlation analysis showed a positive association between the scope for growth and the feeding variables, which was highly significant ( $r > 0.9784$ ,  $P < 0.0001$ ), whereas no association was noted between the oxygen consumption rates ( $r = -0.08$ ,  $P = 0.3579$ ). Correlation of the scope for growth with excretion rates was negative ( $r = -0.2422$ ,  $P = 0.005$ ). The factorial analysis of variance showed that the growth potentials of the two scallops was significantly influenced

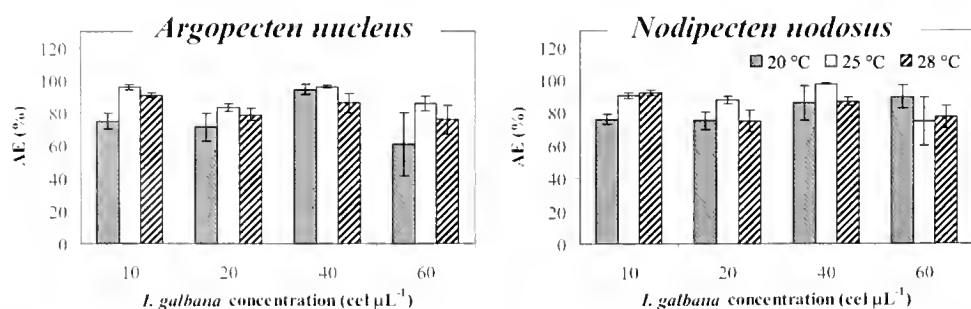


Figure 2. *Argopecten nucleus* and *Nodipecten nodosus*. Absorption efficiencies under different concentrations of *Isochrysis galbana* and water temperatures.

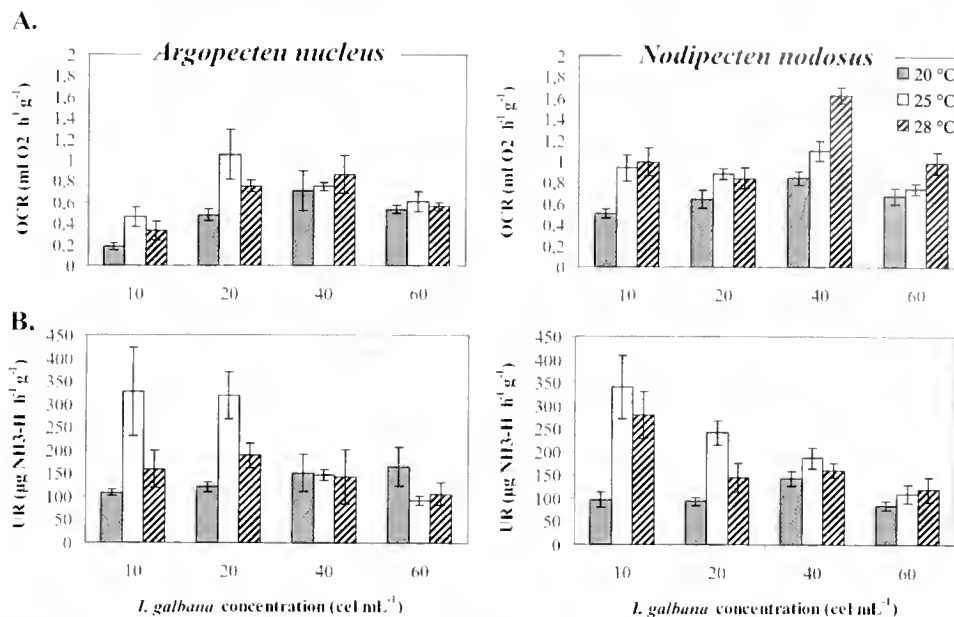


Figure 3. *Argopecten nucleus* and *Nodipecten nodosus*. A. Oxygen consumption rates and B. Ammonia excretion rates under different concentrations of *Isochrysis galbana* and water temperatures.

by the microalgal concentration ( $\text{df} = 3$ ,  $P < 0.0001$ ), whereas the water temperature affected *N. nodosus* ( $\text{df} = 2$ ,  $F = 16.01$ ,  $P = 0.0001$ ) and not *A. nucleus* ( $\text{df} = 2$ ,  $F = 0.86$ ,  $P = 0.4289$ ). The scope for growth increased together with food concentration up to 40 cells  $\mu\text{L}^{-1}$  and thereafter decreased at 60 cells  $\mu\text{L}^{-1}$ , except in *N. nodosus* at the low temperature (20 °C), at which the scope for growth continued to increase. Temperature affected the scope for growth of *N. nodosus* only at the intermediate food concentrations of 20 y 40 cells  $\mu\text{L}^{-1}$  ( $\text{df} = 3$ ,  $P < 0.0277$ ), with an increase in scope for growth stimulated at 25 °C. The analysis of covariance showed that the scope for growth of the two scallops was statistically similar ( $\text{df} = 1$ ,  $F = 3.2$ ,  $P = 0.0761$ ).

## DISCUSSION

### Feeding Rates

The highest rates of filtration, ingestion, and absorption recorded for the two scallop species in this study were obtained at intermediate concentrations of microalgae offered as food. These results were in agreement with results of previous studies on the feeding of bivalves exposed to broad ranges of algal concentration

as in the case of *Mytilus chilensis* (Velasco & Navarro 2002, 2003). The decrease in feeding rates at high concentrations of microalgae has not been noted in other studies, such as those of *Aulacomya ater* (Griffiths & King 1979), *Mytilus chilensis* (Navarro & Winter 1982), *Placopecten magellanicus* (Bacon et al. 1998) and *Musculista senhousia* (Inoue & Yamamuro 2000). Alternatively, stabilization of feeding rates may occur at high food concentrations, as shown for *Tapes philippinarum* (Coutteau et al. 1994); *Mya arenaria* (Bacon et al. 1998) and *Mulinia edulis* (Velasco & Navarro 2002, 2003). It may be that the latter two results were because of the narrow range of microalgal concentrations tested. The reduced feeding rates of the bivalves at low food concentrations has been explained as the direct, and uncontrolled scarcity of food, whereas at high concentrations it may represent a reduction in the pumping of water, and to the increase in production of pseudofeces, which allows the bivalve to regulate the ingestion rate and avoid saturation of the alimentary system (Iglesias et al. 1996, Velasco & Navarro 2002). The concentration of *Isochrysis galbana* at which the ingestion rate of *Argopecten nucleus* and *Nodipecten nodosus* was at a maximum, inducing regulation, or lowering of ingestion ("saturation concentration"), was at 40 cells  $\mu\text{L}^{-1}$  (8 mg  $\text{L}^{-1}$ ); this value coincided with that observed in other bivalves fed on microalgal diets including *Meretrix meretrix*

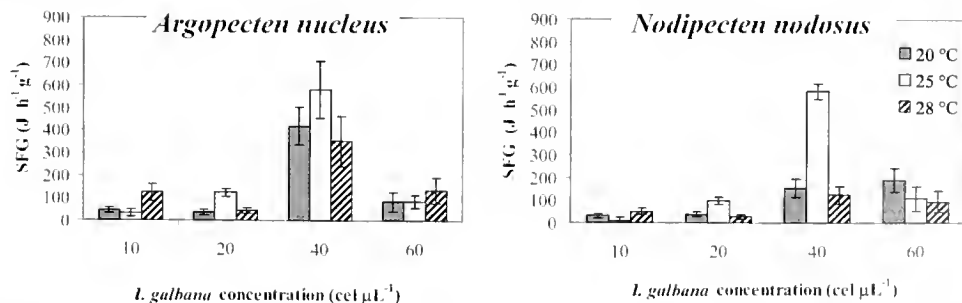


Figure 4. *Argopecten nucleus* and *Nodipecten nodosus*. Scope for growth under different concentrations of *Isochrysis galbana* and water temperatures.

(8 mg L<sup>-1</sup>, Zhuang & Wang 2004) and *P. magellanicus* (7 mg L<sup>-1</sup>, Bacon et al. 1998).

The lack of influence of the temperature on the feeding rates of *Argopecten nucleus* showed that this species had a high capacity for rapid acclimation within the temperature range tested. Similar results had previously been presented for *Argopecten purpuratus* exposed to 16°C and 20°C for three weeks (Navarro et al. 2000). The increase in feeding rates in *Nodipecten nodosus* with increase in temperature and its decrease at temperatures as high as 28°C, indicated that this species did not become acclimated to different temperatures in a short time (15 h). This result coincided with the responses described for other bivalves, including *Argopecten ventricosus* (Sicard et al. 1999) and *Pecten maximus* (Laing 2000). Other studies only described increases in feeding rates with increases in temperature, as with *Arctica islandica*, *Modiolus modiolus* (Winter 1970), *Venerupis decussata*, *Mercenaria mercenaria* (Walne 1972), *Ostrea edulis* (Beiras et al. 1995) and *Pecten fumatus* (Heasman et al. 1996) possibly because of the fact that the cited authors worked within a narrower temperature range. The presently described effect of temperature on the feeding rates of *N. nodosus* was observed only at intermediate food concentrations (20 and 40 cells µL<sup>-1</sup>). Extreme concentrations apparently produce a limiting effect, which annulled the positive effect obtained at intermediate temperature (25°C).

The similarity between feeding rates in *Argopecten nucleus* and *Nodipecten nodosus* contrast with the higher capacity for retaining particulate in the bigger branchial area per unit dry weight of *N. nodosus* (Velasco, in press). It suggests that *A. nucleus* has higher pump activity than *N. nodosus*. Other studies, which have compared interspecific feeding rates have demonstrated greater particle retention and feeding rates in species having greater branchial surface areas (Foster-Smith 1976, Hawkins et al. 1990, Velasco & Navarro 2002).

#### Absorption Efficiency

Absorption efficiency generally decreases when the concentration of microalgae increases, as in the cases of *Mytilus edulis* (Widdows 1978), *Aulacomya ater* (Griffiths & King 1979), *Mytilus chilensis* (Navarro & Winter 1982), *Argopecten purpuratus* (Fernández-Reiriz et al. 2005) and *Hiatella arctica* (Sejr et al. 2004). This has been attributed to the decrease in residence time of the food in the stomach when the ingestion rate is high (Bayne et al. 1989). The absorption efficiencies of *Argopecten nucleus* and *Nodipecten nodosus* were relatively high, and its variation showed no relation with the concentration of microalgae, nor with the ingestion rate, in agreement with the results obtained with *Mya arenaria*, *Placopecten magellanicus* (MacDonald et al. 1998) and in *Meretrix meretrix* (Zhuang & Wang 2004). The maintenance of high absorption efficiencies in *A. nucleus* and *N. nodosus* at the highest food concentration tested could be related to the strong regulation of the ingestion rate, which avoids oversaturation of the digestive tract and affects the efficiency of the digestive process.

The lack of influence of temperature on the absorption efficiency of *Nodipecten nodosus* coincides with that found for *Argopecten purpuratus* after an acclimation period (Navarro et al. 2000), indicating a high degree of plasticity in this variable with regard to temperature changes. In *Argopecten nucleus* the increase in temperature from 20°C to 25°C produced an increase in the absorption efficiency as noted for *Ostrea edulis* (Beiras et al. 1995) and *Meretrix meretrix* (Zhuang & Wang 2004). Low temperatures

produce a decrease in kinetic energy of molecules, and a decrease in the probability that they will react upon colliding (Eckert et al. 1990), which should retard hydrolysis of different substrates and reduce the efficiency of their digestion.

The similarity of the absorption efficiencies of *Argopecten nucleus* and *Nodipecten nodosus* agree with results obtained in comparisons of absorption efficiencies among bivalve species, including *Mulinia edulis* and *Mytilus chilensis* (Velasco & Navarro 2003, 2005) and *Mya arenaria* and *Placopecten magellanicus* (MacDonald et al. 1998). This indirectly suggests a similarity in the composition and/or activity of the digestive enzymes of the two species, possibly induced by being exposed to similar environmental conditions. It was demonstrated in *M. edulis* and *M. chilensis* that although the composition and activity of the digestive enzymes in their digestive glands and crystalline styles were different (Labarta et al. 2002), they had comparable absorption efficiencies (Navarro et al. 2003, Velasco & Navarro 2003).

#### Oxygen Consumption

Oxygen consumption by *Argopecten nucleus* and *Nodipecten nodosus* increased with the increase in microalgae concentration, and at the highest concentration tested, this rate decreased, demonstrating a similar response to that of the feeding rate. This showed that the costs of feeding activities, digestion and absorption were variable and depended on the food concentration. Similar results have been described for other bivalves such as *Donax vittatus* (Ansell 1973), *Aulacomya ater* (Griffiths & King 1979), *Mytilus edulis* (Thompson & Bayne 1972, Bayne et al. 1984, 1989), *Mya arenaria* (MacDonald et al. 1998), *Musculista senhousia* (Inoue & Yamamuro 2000), *Mulinia edulis*, *Mytilus chilensis* (Velasco & Navarro 2003), *Cerastoderma edule* (Navarro et al. 1992, 1994) and *Hiatella arctica* (Sejr et al. 2004). It is in contrast, however, with the finding that there was no influence of food concentration on oxygen consumption in *M. arenaria* and *Placopecten magellanicus* (MacDonald et al. 1998).

The increase in oxygen consumption by *Argopecten nucleus* and *Nodipecten nodosus* with increase in water temperature is explained by the positive effect of increase in temperature on the speeds of metabolic reactions (Eckert et al. 1990) and is in agreement with results from other bivalves such as *Ostrea edulis* (Beiras et al. 1995) *Crassostrea gigas* (Bougrier et al. 1995) and *Argopecten ventricosus* (Sicard et al. 1999). This behavior differs from that found for *O. edulis* (Beiras et al. 1995) and *Argopecten purpuratus* (Navarro et al. 2000) after being experimentally held at a different temperature for a relatively long period (3 wk); under these conditions the oxygen consumption did not vary with temperature, showing an acclimation of the organisms to manipulation of this parameter. According to Eckert et al. (1990), acclimation of tissues and organisms to different temperatures could occur because of the modification of sensitivity of enzymatic activity, changes in molecular structure of one or more enzymes, or to changes in the quantities of enzymes.

In spite of the similarities among feeding rates of the scallops, *Nodipecten nodosus* had greater oxygen consumption than *Argopecten nucleus*, suggesting that the latter species was more efficient than the former in utilization of this resource in carrying on its metabolic functions. In studies comparing oxygen consumption among bivalve species (MacDonald et al. 1998, Velasco & Navarro 2003), it was noted that the existence of interspecific differences in oxygen consumption coincided with parallel differences in feeding rates.

### Excretion Rate

The decrease in excretion rate in *Argopecten nucleus* and *Nodipecten nodosus* with increases in food concentration contrasts with most comparable studies, as the more typical response has been a positive relation between these variables (*Mulinia edulis*, *Mytilus chilensis*, Velasco & Navarro 2003, *Hiatella arcaica*, Sejr et al. 2004), or even no relation at all (*Mya arenaria* and *Placopecten magellanicus*, MacDonald et al. 1998). It is known that bivalves use proteins stored in body tissues as energy sources when the food supply is incapable of satisfying metabolic requirements, resulting in an increase in the excretion rate (Bayne & Newell 1983). Therefore the lower ranges of food concentration tested in this study (10 and 20 cells  $\mu\text{L}^{-1}$ ) may have been insufficient to supply the energetic demands of the two scallop species, which then had to rely on the catabolism of endogenous proteins.

The reduced excretion rates of *Argopecten nucleus* and *Nodipecten nodosus* at low water temperatures, particularly when the food concentration was 20 cells  $\mu\text{L}^{-1}$  or less, could be explained by a decrease in metabolism under these conditions, which could in turn, lower the metabolic demands of the organisms to a point at which they were not required to resort to the use of stored proteins. The same tendency has been observed in other bivalves such as *Ostrea edulis* (Beiras et al. 1995).

The excretion rate of *Argopecten nucleus* was similar to that of *Nodipecten nodosus*, similar to findings in interspecific comparisons under the same conditions in *Mya arenaria* and *Placopecten magellanicus* (MacDonald et al. 1998) and *Mulinia edulis* and *Mytilus chilensis* (Velasco & Navarro 2003). The similarity in excretion rates of the bivalves, together with the similarities between absorption rates, suggested that the utilization of proteins in the two species comparisons were quite similar.

### Scope for Growth

The positive high correlation between scope for growth and feeding rates, and the low values of oxygen consumption and excretion indicated that the energy acquisition by feeding was more important than the energy output in determining the values of scope for growth. Because values for ingestion and absorption depend on the filtration rate, it can be assumed that the filtration rate is the main determinant of physiological condition in *Argopecten nucleus* and *Nodipecten nodosus* under the conditions tested in the present study. Similar conclusions have been derived for *Argopecten purpuratus* (Navarro et al. 2000), *Mya arenaria* and *Placopecten magellanicus* (Bacon et al. 1998, MacDonald et al. 1998), and *Mulinia edulis* and *Mytilus chilensis* (Velasco & Navarro 2002; 2003). Nevertheless, in species such as *Ostrea edulis* (Ansell & Sivadas 1973) and *M. edulis* (Velasco & Navarro 2002), under certain conditions the oxygen consumption may constitute an important portion of the energy absorbed, and may have an important effect on the growth potential.

The high growth potentials of *Argopecten nucleus* and *Nodipecten nodosus* when fed intermediate microalgal concentrations (40 cel  $\mu\text{L}^{-1}$ ), as well as the low values obtained at the extremes of the algal concentration (10 and 60 cel  $\mu\text{L}^{-1}$ ), are in accord with results obtained for *Aulacomys ater* (Griffiths & King 1979), and *Mytilus chilensis* (Velasco & Navarro, 2003). They differ, however, with findings from other studies, where scope for growth and algae concentration showed an inverse relation, as in *M. chilensis* (Navarro & Winter 1982), or a direct relation as in *Cerastoderma edule*, *Tapes decussatus* (Navarro & Iglesias, 1995), *Mulinia edu-*

*lis* (Velasco & Navarro 2003), *Mya arenaria* and *Placopecten magellanicus* (MacDonald et al. 1998). These differences in the responses of the scope for growth to food concentration may be caused by the utilization of more narrow ranges of food concentration in the laboratory than those to which the test organisms are accustomed in their natural habitats.

The scope for growth of *A. nucleus* was not influenced by temperature, showing that this species had great plasticity of physiological rates in relation to this factor. On the other hand, the scope for growth of *N. nodosus* was greater at the intermediate temperature in our study (25°C), where there was a high degree of sensitivity of the feeding rates in relation to temperature. The lowering of scope for growth at lower temperature was probably related to decrease in metabolic rate, and at high temperatures to the denaturation of related enzymes (Eckert et al. 1990). The direct relation between scope for growth and temperature coincide with that observed in other species such as *Cerastoderma edule*, *Tapes decussatus* (Navarro & Iglesias 1995), *Argopecten purpuratus* (Navarro et al. 2000) and *Pecten maximus* (Laing 2000). At extremes of food concentration, the scope for growth of *Nodipecten nodosus* was low, and independent of temperature, therefore under these conditions the negative effects of either the absence, or excess of food on the physiological condition of the organism nullified the positive effect of providing an optimal temperature of 25°C. Navarro and Iglesias (1995) found that at very low concentrations of food that the scope for growth of *Cerastoderma edule* and *Tapes decussatus* became negative and decreased with increase in temperature. In this study, the food concentrations were not so extreme as to produce negative scope for growth at which we might have observed a similar phenomenon.

It has been found that the optimal food concentration increases with the water temperature for *Arctica islandica*, *Modiolus modiolus* (Winter 1970) and *Pecten maximus* (Laing 2000). This relation was not observed in this study with either scallop species. In *Argopecten nucleus* the optimal food concentration was independent of temperature, because the optimal food concentration was found to be the same (40 cel  $\mu\text{L}^{-1}$ ) at all the temperatures tested. Conversely, in *Nodipecten nodosus* the optimal food concentration increased with decrease in temperature. This response was probably because saturation of the alimentary system did not occur under low temperature conditions and high food concentrations, thus permitting continuance of high ingestion and absorption rates.

Similarities between the scope for growth in *Argopecten nucleus* and *Nodipecten nodosus* indicate that the two species may compete with equal efficiency under the same conditions of food concentration and temperature, based on the environmental conditions occurring in each of their natural habitats. Studies that have compared scope for growth between species with differing habitats (epifaunal and infaunal) have found interspecific similarities under certain shared feeding conditions, which may not occur under other conditions in which interspecific differences become manifest (MacDonald et al. 1998, Velasco & Navarro 2002, Savina & Pouvreau 2004).

In conclusion, this study found that: (1) the scope for growth and the feeding rates of *Argopecten nucleus* and *Nodipecten nodosus* increased with increased food concentration but decreased at higher and lower extremes of food concentration; (2) the scope for growth and feeding rates of *A. nucleus* were not affected by temperature, with the reverse true for *N. nodosus*, increasing at 25°C and decreasing at higher temperature (28°C); (3) *A. nucleus* and *N. nodosus* showed similar responses and values in all their physi-



ological rates, except for oxygen consumption, where *A. nucleus* displayed lower rates.

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## SEQUENCE ANALYSIS OF THE RIBOSOMAL DNA INTERNAL TRANSCRIBED SPACERS AND 5.8S RIBOSOMAL RNA GENE IN REPRESENTATIVES OF THE CLAM FAMILY VENERIDAE (MOLLUSCA: BIVALVIA)

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**ABSTRACT** The first and second internal transcribed spacer (ITS1 and ITS2) regions of the ribosomal DNA from six species, *Meretrix meretrix*, *Cyclina sinensis*, *Mercenaria mercenaria*, *Protothaca jadoensis*, *Dosinia corrugata* and *Ruditapes philippinarum*, in the family Veneridae were PCR amplified and sequenced. The size of the ITS1 sequence ranged from 522–900 bp, which was the largest range so far reported for a bivalve species, with GC contents from 57.66% to 65.62%. The size of the ITS2 sequence ranged from 281–412 bp, with GC contents from 65.21% to 67.87%. Extensive sequence variation and obvious length polymorphisms were noted for both regions in these species, and ITS2 sequence similarity was higher than that of ITS1 across species. The complete sequences of 5.8S ribosomal RNA gene were obtained by assembling ITS1 and ITS2 sequences, and the sequence length in all species was 157 bp. The phylogenetic tree of Veneridae clams was reconstructed by using ITS2 containing partial sequences of both 5.8S and 28S rDNA, and corresponding sequence information in *Arctica islandica* (Dahlgren et al. 2000) as an outgroup species. Tree topologies indicated that *P. jadoensis* has a closer relationship with *M. mercenaria* than with other species.

**KEY WORDS:** Veneridae, rDNA, internal transcribed spacer, 5.8S rRNA gene, phylogenetic analysis, species identification

### INTRODUCTION

The internal transcribed spacers (ITSs) of nuclear ribosomal DNA (rDNA) is one of the most extensively sequenced molecular markers, and the region is a component of a rDNA cistron, which consists of 18S, ITS1, 5.8S, ITS2 and 28S. ITSs exist in several hundred copies in most eukaryotes. They are located in one or several loci and distributed in one or several chromosomes. The nuclear rDNA copies within a genome can be highly homogeneous because of concerted evolution of intra and interchromosomal loci. ITS1 and ITS2 are noncoding regions located in rDNA between 18S and 5.8S rRNA genes, and between 5.8S and 28S rRNA genes, respectively (Insua et al. 2003, Jansen et al. 2006, Won & Renner 2005).

Because ITSs sequences show more divergence than their flanking coding regions and are easily amplified, they are routinely used to distinguish related species and to infer phylogenetic relationships from populations to families and even higher taxonomic levels (Coleman & Vacquier 2002). In bivalve mollusks, a variety of methods, such as PCR amplification alone, PCR amplification followed by restriction analysis or sequencing was used to differentiate related species (Ding et al. 2004, Fernández et al. 2001, Insua et al. 2003, Kenchington et al. 2002, López-piñón et al. 2002, Yu et al. 2000) and exploring the phylogenetic relationship (He et al. 2005, King et al. 1999, Vidigal et al. 2000, Vidigal et al. 2004, Yu et al. 2001) among bivalve species.

The family Veneridae is a group of bivalve mollusks prevalent in seawater all over the world. There are more than 500 species, many of which are commercially valuable and ecologically crucial because of their dominance in benthic communities. A study of the phylogenesis of Veneridae was carried out using partial sequence of the mitochondrial 16S rRNA gene (Canapa et al. 2003).

The purpose of this study is to amplify and sequence the ITS1 and ITS2 regions of 6 Veneridae species, *Meretrix meretrix*, *Cyclina sinensis*, *Mercenaria mercenaria*, *Protothaca jadoensis*,

*Dosinia corrugata* and *Ruditapes philippinarum*, to provide the basic characteristics of these sequences and to assess the similarity among species. The results from this study will provide useful information in species identification and phylogenetic analysis of Veneridae clams.

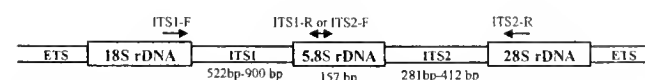
### MATERIALS AND METHODS

#### Sample Collection and DNA Extraction

Specimens of *Meretrix meretrix* and *Cyclina sinensis* were collected from Dalian, Liaoning province, China, and *Mercenaria mercenaria*, *Protothaca jadoensis*, *Dosinia corrugata* and *Ruditapes philippinarum* belonging to five subfamilies of Veneridae, from Lianyungang, Jiangsu province. Total genomic DNA was extracted from approximately 50 mg of adductor muscle following a modified CTAB protocol (Winnepenninckx et al. 1993). Briefly, the tissue was incubated for 15 min at 55°C in 600 µl CTAB buffer containing 25 µl 10 mg/mL proteinase K, homogenized with a pestle, and incubated for an additional 60 min. After extractions with saturated phenol and then chloroform: isoamyl alcohol (24:1), genomic DNA was ethanol-precipitated, resuspended in 50-µl TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and stored at –20°C for future use.

#### PCR Amplification and Sequencing

ITS1 region was amplified using ITS1-F (5'-GGTGAACCT-GCGGATGGA-3') and ITS1-R (5'-GCTGGCTGCGCTCTTCAT-3') as primers, which anneal to the 3' end of 18S ribosomal RNA gene and the 5.8S ribosomal RNA gene, respectively. ITS2



**Figure 1.** Approximate locations of primers used for ITS1 and ITS2 amplification. Numbers below boxes refer to numbers of base pairs, ITS1 = first internal transcribed spacer, ITS2 = second internal transcribed spacer, ETS = external transcribed spacer

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Figure 2. Continued

were selected by 2% w/v agarose gels electrophoresis. The ITS1 and ITS2 regions were PCR amplified from at least 10 individuals from each of six species, but only two PCR products were selected and delivered to Shanghai Sangon Biological Engineering & Technology and Service Co. Ltd. (Shanghai, China) for sequence. The sequence was conducted in both forward and reverse directions using the amplification primers by ABI prism 377 automatic DNA sequencer.

The forward and reverse sequences were assembled using SeqManII software in DNASTar Package version 5.01 to obtain ITS1 and ITS2 sequences, and each of the different sequences was registered as an ITS1 or ITS2 haplotype in GenBank. Each newly determined sequence was checked against existing haplotypes using DNASTar and then the sequence was registered as a new haplotype. Because ITS1-R and ITS2-F primers anneal to the same region in

5.8S rDNA (Fig. 1), ITS1 and ITS2 can be assembled into an ITS, and the complete sequence of 5.8S ribosomal RNA gene can be obtained. The boundaries of the coding and spacer regions were determined by comparison with the sequence information of *Arctica islandica* (GenBank accession No. AF 202106). The sequences were edited and analyzed using the program EditSeq. ITS sequences containing the 5.8S ribosomal RNA gene across six species were aligned using program MegAlign of DNASTar package using the Clustal W method. The trees were produced by Neighbor-Joining (NJ) and Maximum parsimony (MP) methods using MEGA software Version 3.1 (Kumar et al. 2004).

## RESULTS

The ITS1 and ITS2 regions were PCR amplified and sequenced among six species, the ITS1 and ITS2 were assembled into a single ITS sequence. For *M. meretrix*, *M. mercenaria*, *D. corrugata* and *R. philippinarum*, the identical ITS sequences were detected between 2 individuals within a species and named haplotypes WD1, YL1, BL1, FL1, respectively. For other species, *C. sinensis* and *P. jedoensis*, the ITS sequence were different between 2 individuals within a species and were named haplotypes QD1, QD2 for *C. sinensis*, and JL1, JL2 for *P. jedoensis*. Figure 2 showed the alignment of the ITS sequences including 5.8S rDNA, spanning 18S and 28S rDNA among eight haplotypes from six Veneridae species, and Table 1 displayed the length and GC contents of ITS1, 5.8S ribosomal DNA, and ITS2, and GenBank accession number.

### Sequence Analysis of the First Internal Transcribed Spacer

The length of ITS1 sequence ranged from 522 bp to 900 bp, and GC contents from 57.66% to 65.62% in six species (Table 1). As seen in Table 1 interspecific ITS1 sequences showed remarkable divergence and obvious length polymorphism. The GC contents in ITS1 were higher than AT contents in all species. *M. meretrix* had the longest ITS1 sequence (900 bp) with 61.67% GC contents, and two trinucleotide microsatellites (AGT)<sub>n</sub> were observed (Fig. 2). As well, a repeat sequence (AGT)<sub>n</sub> GAAAAA(G)GCGAAG-GAGCCGCTGGCCCTTC was found occurring twice in ITS1 region in this species. *D. corrugata* had the shortest ITS1 sequence (522 bp) with 57.66% GC contents. *P. jedoensis*, *M. mercenaria*, *R. philippinarum* and *C. sinensis* had intermediate ITS1 size, 864 bp, 679 bp, 632 bp and 585 bp, respectively. A dinucleotide microsatellite (TA)<sub>n</sub> was observed in the ITS1 region of *C. sinensis* (Fig. 2).

ITS1 sequences of six species were aligned. The percentages of sequence divergence and identity were showed in Table 2. Inter-

specific ITS1 sequence divergences were very high, because of the presence of transition, transversion and indels (insertions/deletions). The percentages of interspecific sequence divergence were from 27.2% (*M. mercenaria* and *P. jedoensis*) to 102.6% (*M. meretrix* and *R. philippinarum*). *P. jedoensis* exhibited 0.1% intraspecific sequence divergence between two individuals. For other species, *M. meretrix*, *C. sinensis*, *M. mercenaria*, *D. corrugata* and *R. philippinarum*, the ITS1 sequence was identical between two individuals within a species.

We found one relatively conserved motif in the ITS1 region across the six species of Veneridae (Fig. 2), with a size of 54 bp showed 18 polymorphic sites, 12 of which were transition and six were transversion.

### Sequence Analysis of the Second Internal Transcribed Spacer

The length of ITS2 sequences ranged from 281 bp to 412 bp and GC contents from 65.21% to 67.87% (Table 1). The interindividual variation for ITS2 was detected in both *P. jedoensis* and *C. sinensis*. In contrast, *M. meretrix*, *M. mercenaria*, *D. corrugata* and *R. philippinarum* had the same ITS2 sequences in two individuals. The GC contents of ITS2 sequences were higher than AT contents in all species. *M. meretrix* had the longest ITS2 sequence (412 bp) with 65.29% GC content. *P. jedoensis* had the shortest ITS2 sequence (281 bp to 282 bp) with 67.38% to 67.62% GC contents; *C. sinensis*, *M. mercenaria*, *D. corrugata* and *R. philippinarum* had intermediate ITS2 sequence (Table 1). A quadrinucleotide microsatellite (AGCG)<sub>n</sub> in *M. meretrix* and three dinucleotide microsatellites (AG)<sub>n</sub> in *M. meretrix*, *R. philippinarum* and *D. corrugata* were observed (Fig. 2).

ITS2 regions from 8 haplotypes in six species were aligned. Percentages of sequence divergence and identity were listed in Table 3. ITS2 showed remarkable interspecific sequence divergences and obvious length polymorphism but to less compared with ITS1. Percentages of interspecific sequence divergence were from 21.6% (*P. jedoensis* and *M. mercenaria*) to 80.7% (*M. meretrix* and *C. sinensis*). Intraspecific sequence divergences in ITS2 region between two individuals were detected in *C. sinensis* (0.5%) and *P. jedoensis* (1.1%). For other species, *M. meretrix*, *M. mercenaria*, *D. corrugata* and *R. philippinarum*, the ITS2 sequence was identical between two individuals within a species.

We also detected two relatively conserved motifs in ITS2 region across six species (Fig. 2). The first relatively conserved motif with a size of 31 bp displayed five transition or transversion polymorphic sites, and the second with a size of 24 bp showed three transition or transversion polymorphic sites.

TABLE 1.  
The length and GC contents of ITS1, 5.8S ribosomal DNA, and ITS2 in eight haplotypes from six species

Species (Sample size)	Haplotype	ITS1		5.8S rDNA		ITS2		GenBank accession number
		Length (bp)	GC (%)	Length (bp)	GC (%)	Length (bp)	GC (%)	
<i>Meretrix meretrix</i> (2)	WD1	900	61.67	157	58.60	412	65.29	DQ132788, DQ191390
<i>Cyclina sinensis</i> (2)	QD1	585	61.03	157	58.60	386	66.06	DQ132787, DQ191387
	QD2	585	61.03			388	65.21	DQ132787, DQ191388
<i>Mercenaria mercenaria</i> (2)	YL1	679	63.03	157	57.96	361	67.87	DQ132789, DQ191391
	JL1	864	65.62			282	67.38	DQ220290, DQ191389
<i>Protothaca jedoensis</i> (2)	JL2	864	65.51	157	57.96	281	67.62	DQ220291, DQ132791
<i>Dosinia corrugata</i> (2)	BL1	522	57.66	157	57.96	294	66.33	DQ346656
<i>Ruditapes philippinarum</i> (2)	FL1	632	63.92	157	57.96	379	67.28	DQ399404

TABLE 2.

Percentage of sequence divergence (below triangle) and identity (above triangle) of ITS1 sequences across seven haplotypes from six species by Clustal W method

Species	1	2	3	4	5	6	7
1 <i>Meretrix meretrix</i>	***	31.8	31.8	28.7	28.8	31.0	27.2
2 <i>Cyclina sinensis</i> QD1	91.7	***	35.4	33.5	33.5	35.6	30.8
3 <i>Mercenaria mercenaria</i>	96.8	46.9	***	45.4	45.1	48.5	30.2
4 <i>Protothaca jedoensis</i> JL1	88.9	50.4	27.2	***	99.9	41.8	32.0
5 <i>Protothaca jedoensis</i> JL2	88.4	50.8	27.4	0.1	***	41.8	32.0
6 <i>Dosinia corrugata</i>	96.0	43.5	28.2	40.6	40.2	***	31.2
7 <i>Ruditapes philippinarum</i>	102.6	63.3	58.4	52.1	52.5	66.0	***

#### Sequence Analysis of the 5.8S Ribosomal RNA Gene

Through assembling ITS1 and ITS2 sequences, we obtained complete sequences of the 5.8S ribosomal RNA gene of six Veneridae species. The length of the 5.8S ribosomal RNA gene was 157 bp in all species (Fig. 2), and the GC contents ranged from 57.96% to 58.60% depending on species. The sequence divergences ranged from 0.0% to 6.0% across six species (Table 4). The 5.8S rRNA genes contained 10 polymorphic sites in these species, four of which were transition and six were transversion. Moreover, *P. jedoensis*, *M. mercenaria*, *D. corrugata* and *R. philippinarum* showed identical 5.8S rDNA sequence.

#### Phylogenetic Analysis in Terms of ITS2 Containing Partial 5.8S and 28S Ribosomal RNA Gene Sequences

Using ITS2 spanning 5.8S and 28S rDNA sequences as a molecular marker, the phylogenetic tree of Veneridae clam was constructed using *Arctica islandica* (GenBank accession number AF202106) as an outgroup species with Neighbor-Joining (NJ) and Maximum parsimony (MP) methods (Fig. 3).

The topology of the tree by MP method was similar to that by NJ method. Tree topologies showed that six Veneridae species were in the same group and forming a clade. In Veneridae, *P. jedoensis* was first grouped with *M. mercenaria* with a high bootstrap value of 94% in MP tree and 99% in NJ tree and then formed a clade with *D. corrugata* with a lower bootstrap value of 66% in MP tree and 80% in NJ tree; in contrast, *M. meretrix*, *C. sinensis* and *R. philippinarum* formed a monophyletic group. This indicated that *P. jedoensis* and *M. mercenaria* has a closer relationship than other species.

In contrast to ITS2, the construction of phylogeny using ITS1 was not applicable because of some disadvantages, such as ITS1 length variation, the presence of tandem repeated sequences, and

large number of indels. ITS1 sequences analysis, using MP and NJ methods, gave poor resolution in genus level. Therefore ITS1 was not used for phylogenetic analysis.

#### DISCUSSION

The current study provided information about the nucleotide sequences of ITS1 and ITS2 regions, complete sequence of the 5.8S ribosomal RNA gene in six Veneridae clams (*M. meretrix*, *C. sinensis*, *M. mercenaria*, *P. jedoensis*, *D. corrugata* and *R. philippinarum*). Their characteristics and variation were demonstrated through PCR amplification and sequencing. The length of ITS1 in the family Veneridae were longer than that in four Pectinidae scallop with size 209 bp to 276 bp for ITS1 and 270 bp to 294 bp for ITS2 (Insua et al. 2003), respectively. It should be noted that the length range in ITS1 reported in the current study was among the largest observed in bivalves. The size of ITS is species-dependent and the difference could be significant among species. The largest ITS1, such as that in Ladybird beetle *Exocomus quadripustulatus*, was as long as 2572 bp (Von der Schulenburg et al. 2001), and the shortest one only 70 bp to 80 bp in *Acropora* species (Odorico & Miller 1997). Depending on species, the length of ITS2 can be twice or more that of ITS1 (Dahlgren et al. 2000, Fernández et al. 2001, Yu et al. 2000), both situation, the length of ITS1 similar to ITS2 or larger than ITS2 were also reported (Chen et al. 2002, Coleman & Vacquier 2002). The GC contents ranged from 57.66% to 65.62% for ITS1 and from 65.21% to 67.87% for ITS2 in Veneridae tested in the current study. These species had higher GC contents than other bivalve species. For instance, the GC contents in Pectinidae scallop were 43% to 49% for ITS1 or 44% to 49% for ITS2 (Insua et al. 2003) and 51.9% to 55.5% for ITS2 in Pearl Oyster (He et al. 2005). A frequent characteristic of spacers is a balanced GC content between ITS1 and ITS2, and this

TABLE 3.

Percentage of sequence divergence (below triangle) and identity (above triangle) of ITS2 sequences across eight haplotypes from six species by Clustal W method

Haplotypes	1	2	3	4	5	6	7	8
1 <i>Meretrix meretrix</i>	***	35.8	36.5	34.3	37.8	39.0	39.7	33.2
2 <i>Cyclina sinensis</i> QD1	80.7	***	96.6	40.1	39.9	41.8	37.6	39.2
3 <i>Cyclina sinensis</i> QD2	79.7	0.5	***	40.3	39.6	42.6	37.6	37.9
4 <i>Mercenaria mercenaria</i>	61.3	56.4	56.7	***	53.0	52.8	62.4	39.2
5 <i>Protothaca jedoensis</i> JL1	46.8	48.1	47.6	22.6	***	97.2	50.9	42.4
6 <i>Protothaca jedoensis</i> JL2	47.0	49.2	48.6	21.6	1.1	***	52.1	42.9
7 <i>Dosinia corrugata</i>	55.2	52.6	53.0	27.0	24.5	23.3	***	44.1
8 <i>Ruditapes philippinarum</i>	72.8	72.0	71.8	51.4	47.6	47.1	49.8	***

TABLE 4.

Percentage of sequence divergences (below triangle) and base variation (above triangle, transitions/transversions) of 5.8S rDNA across six species by Clustal W method

Species	1	2	3	4	5	6
1 <i>Meretrix meretrix</i>	***	9 (3/6)	7 (3/4)	7 (3/4)	7 (3/4)	7 (3/4)
2 <i>Cyclina sinensis</i>	6.0	***	4 (2/2)	4 (2/2)	4 (2/2)	4 (2/2)
3 <i>Mercenaria mercenaria</i>	4.6	2.6	***	0 (0/0)	0 (0/0)	0 (0/0)
4 <i>Protothaca jedoensis</i>	4.6	2.6	0.0	***	0 (0/0)	0 (0/0)
5 <i>Dosinia corrugata</i>	4.6	2.6	0.0	0.0	***	0 (0/0)
6 <i>Ruditapes philippinarum</i>	4.6	2.6	0.0	0.0	0.0	***

also occurs in the Veneridae spacers, this fact could indicate the coevolution between the two spacers at the level of base composition.

Both ITS1 and ITS2 regions in family Veneridae exhibit extensive sequence variation and obvious length polymorphisms, as is similar to Crustacea *Eriocheir formosa* (Chu et al. 2001) and other bivalves (Ding et al. 2004). In this study, the construction of topology in Veneridae clam using ITS1 sequence information is not applicable because of the high length variation, the presence of tandem repeated sequences and large number of indels in ITS1. However, it may be useful in phylogenetic analysis at lower taxonomic levels, such as subspecies, breeds or population levels. In contrast, the interspecific ITS2 sequence similarity in these species were higher than that of ITS1, and considering the length of ITS2 were shorter than that of ITS1, providing advantage and convenience in designing primers and sequencing. ITS2 is an ideal candidate for the study of genetic structure in Veneridae. The further study on the efficiency for combining the method in the current study with other techniques, such as PCR-RFLP, is promising.

In this study, a relatively conserved motif in ITS1 region and two relatively conserved motifs in ITS2 region were found in these species. This indicated that these motifs might be involved in certain nucleotide acid-related functions, such as in rRNA processing (Insua et al. 2003). Beside, two trinucleotide microsatellites (AGT)<sub>n</sub> in *M. meretrix* and a dinucleotide microsatellite (TA)<sub>n</sub> in *C. sinensis* were found locating in ITS1 region, and a quadrinucleotide microsatellite (AGCG)<sub>n</sub> and three dinucleotide microsatellites (AG)<sub>n</sub> was found locating ITS2 region. These microsatellites may be served as good markers in future studies. In the bivalve species, a dinucleotide microsatellite (GT)<sub>n</sub> and a trinucleotide microsatellite (TAC)<sub>n</sub> were found in ITS sequence of *Lasmigona* (King et al. 1999), but so far there has been no report from Veneridae.

Although interindividual sequence divergences in ITS1 and ITS2 regions were detected in *C. sinensis* (0.3% for ITS2) and *P. jedoensis* (0.1% for ITS1 and 0.6% for ITS2). Because of the presence of polymerase and sequencing error, the sequences cannot be thought as different only if when sequence divergence more than 0.9% (Kong et al. 2002). *C. sinensis* and *P. jedoensis* showed no intraspecific variations in ITS1 and ITS2. The 5.8S ribosomal RNA genes were highly conserved across these species studied in the current study, and had a length of 157 bp, which was reported in other bivalves, such as Pectinidae scallop species (*A. opercularis*, *M. varia*, *H. distortus*, *P. maximus*) (Insua et al. 2003) and *Arctica islandica* (Dahlgren et al. 2000).

The spacer regions, ITS1 and ITS2, of the rDNA are widely and routinely used in analysis of species relationships by using a phylogenetic reconstruction method in various organisms. It was successfully applied in analysis of phylogenetic relationship among the *Biomphalaria* species and among Pearl Oysters, and the conclusions from phylogenetic tree were well in agreement with those from analysis based on morphological systematics and other molecular techniques, such as polymerase chain reaction and restriction fragment length polymorphism analysis (He et al. 2005, Vidigal et al. 2000, Vidigal et al. 2004). Our study demonstrated that ITS1 provided weak phylogenetic signal in discrimination of Veneridae, whereas, ITS2 regions were effective in identifying phylogenetic relationships among Veneridae species. In this study, the tree obtained by ITS2 sequence analysis revealed that *P. jedoensis* and *M. mercenaria*, belonging to subfamilies Chioninae, has a close relationship than other species, belonging to subfamilies Meretricinae, Tapetinae and Cyclininae. Phylogenetic analysis of ITS2 sequences through both methods generated trees with similar topologies that were very concurrent with the morphological taxonomy proposed by Keen. Therefore ITS2 sequence characteristics are an efficient tool in reconstruction of evolutionary

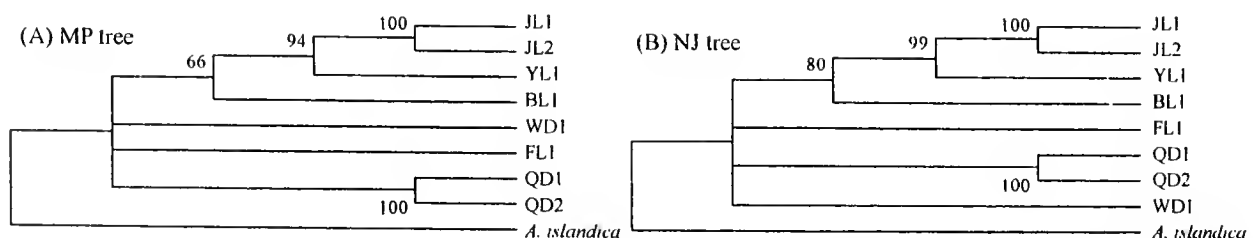


Figure 3. Tree topology constructed in terms of ITS2 spanning 5.8S and 28S rDNA sequences across eight haplotypes from six species of Veneridae, *P. jedoensis* (JL1, JL2), *M. mercenaria* (YL1), *D. corrugata* (BL1), *M. meretrix* (WD1), *R. philippinarum* (FL1) and *C. sinensis* (QD1, QD2), with *Arctica islandica* as an outgroup species using (A) Maximum parsimony (MP) method and (B) Neighbor-Joining method. Numbers represents bootstrap percentages. The topologies were tested using bootstrap analyses (10,000 replicates).



relationship among these organisms, and they can be applied in establishment of species relationship, or reevaluation of the traditional taxonomy.

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## AMPELISCA AMPHIPOD TUBE MATS MAY ENHANCE ABUNDANCE OF NORTHERN QUAHOGS *MERCENARIA MERCENARIA* IN MUDDY SEDIMENTS

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**ABSTRACT** Field surveys in southeastern Raritan Bay and laboratory studies from 1999 to 2005 were conducted to compare the characteristics of mud and sand habitats in relation to the abundance of the northern quahog (*Mercenaria mercenaria*). In 2000, the population density of quahogs was about 15 times higher in the mud habitat than in the sand habitat. In addition, the mud habitat also had a dense population of the amphipod *Ampelisca abdita* (about 24,000 m<sup>-2</sup>) associated with it. This species produces mats of tubes over the bottom. The sediment surface of the mud was comprised mostly of fecal pellets, the majority of which was produced by *A. abdita*. In contrast, the sand habitat did not have *A. abdita* tubes or much erect surface structure; its sediments were comprised of medium grain sand ( $\phi = 1.17$ – $1.4$ ). In southeastern Raritan Bay, the principal quahog predators are the longwrist hermit crab (*Pagurus longicarpus*), Atlantic oyster drill (*Urosalpinx cinerea*), and xanthid mud crabs. Collectively, they were >7 times more abundant in the sand habitat than in the mud habitat. We suggest that quahogs are abundant in the mud habitat because the presence of the tube mats probably reduces water siltation, encourages settlement of larval quahogs and deters predation on the quahogs.

**KEY WORDS:** *Mercenaria*, *Ampelisca*, habitat, pellets, sand, tube mats, predators

### INTRODUCTION

Northern quahogs (*Mercenaria mercenaria*) can be found in estuaries along the Atlantic coast of North America from the Gulf of St. Lawrence to the Florida keys (Harte 2001). In 2004, the United States commercial landings of 9.4 million pounds (meats) of northern quahogs exceeded landings of any other estuarine bivalve; their ex-vessel value was \$37.8 million (Pritchard 2005). Fishermen typically harvest quahogs in sand or muddy-sand sediments, but quahogs are nearly always scarce in soft mud habitats (Rhoads & Young 1970, Rhoads 1974, Fegley 2001, Mann et al. 2005). This scarcity is apparently caused by high silt concentrations in the water close to the bottom (Rhoads & Young 1970, Murphy 1985). The silt clogs the digestive tract of larval quahogs and slows the growth of sedentary quahogs (Davis 1960, Bricelj et al. 1984). Most field studies of quahogs have emphasized the suppressing effects of predators on quahog abundance as summarized by MacKenzie (1977), Kraeuter (2001) and MacKenzie et al. (2002).

In southeastern Raritan Bay, New Jersey, in contrast to the other locations outside of Raritan Bay, quahogs are abundant in mud areas located in the bay's broad central area, and they are far more abundant there than in its sand areas that extend from its shores to the mud area (Celestino, 2003). A major difference between the mud and sand habitats in Raritan Bay is the presence of dense tube mats of the ampeliscid amphipod, *Ampelisca abdita*. *A. abdita* ranges from Maine to at least Florida, and produces dense masses of tubes (Stickney & Stringer 1957, Mills 1967, 1969) (Table 1). For this study, from 1999 to 2003, we examined several features of the mud and sand habitats in southeastern Raritan Bay that may explain the difference in quahog abundances between the two habitats. Particular emphasis was placed on describing the autecology of *A. abdita*, including its distribution, its persistence in the area through time, its number of generations per year and the temporal appearance of its tubes. In the Discussion, we compare our findings with those in the literature on the autecology of the quahog and suggest why the presence of *A. abdita*

may be the reason the quahog abundance is high in the mud area of southeastern Raritan Bay.

### Characteristics of Southeastern Raritan Bay

The deeper areas of southeastern Raritan Bay primarily consist of mud sediments that extend over an area of about 30 km<sup>2</sup> (Fig. 1). The water depth averages about 7 m (range, 3.4–8 m) at low tide. The shallower areas that extend from the shorelines offshore to the mud areas consist primarily of sand sediments and have a total area of about 18 km<sup>2</sup>. At their midpoints from the southern and eastern shores to the edges of the mud areas, water depths over the sand areas range from 3–4 m and 1–2.5 m, respectively, at low tide. Salinities in this part of the bay are about 26‰ during summer and 21‰ to 24‰ during winter (Cerrato et al. 1989). Surface water temperatures average 21°C to 24°C during summer, 0.4°C in early February, and 6°C to 7°C during March.

In the late 1980s and early 1990s, the mud area of southeastern Raritan Bay had relatively few northern quahogs and a large number of starfish (*Asterias forbesi*) (Cerrato et al. 1989, MacKenzie & Pikanowski 1999). By the mid 1990s and thereafter, the starfish were no longer present and the quahogs became abundant (MacKenzie & Pikanowski 1999). In 2000, the State of New Jersey Department of Environmental Protection surveyed the quahog abundance in southeastern Raritan Bay. Its population density was about 15 times higher in the mud than in the sand habitats: 14.9 quahogs m<sup>-2</sup> versus 1.0 quahogs m<sup>-2</sup> (Fig. 1) (Celestino 2003). Since the mid 1990s, southeastern Raritan Bay has had a year-round commercial fishery for quahogs. It consists of 20–100 boats, each with one fisherman using a bull rake. The fishermen harvest quahogs almost exclusively from the mud area.

In 1974, *A. abdita* was scarce in southeastern Raritan Bay (Steimle & Caracciolo-Ward, 1989), but it was abundant and widespread in 1986 in the mud area (Cerrato et al. 1989). It is not known when *A. abdita* became abundant between 1974 and 1986. It is assumed that it was abundant between 1986 and 1999, but there are no data to prove this. *A. abdita* occupy tubes about 3.5 cm long and 2.5–3.5 mm wide at the mouth end. The tubes are flattened laterally, and are composed of nonchitinous, pliable organic material. Each *A. abdita* forms a tube by secreting mucus around

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TABLE 1.  
Abundances of *Ampelisca abdita* in three northeastern United States estuaries.

Location	Number m <sup>-2</sup>	Reference
Long Island Sound	7,000 to 80,450	Sanders et al. (1962)
Jamaica Bay, NY	18,000 to 80,000	Franz and Tanacredi (1992)
Raritan Bay	989 to 57,185	This study

its body. This mucus collects surface sediments on the tube's outer surface as the amphipod forms it. The Raritan Bay tubes are covered with a continuous layer of brown fecal pellets and finer particles held in place by the mucus. The tube lining is smooth and parchment-like, and lacks attached sediments. The top opening of the tube becomes its mouth and the bottom opening is gradually attenuated. About two-thirds of the tube length extends vertically into the water; the remainder is anchored in the sediment (Fig. 2).

Mills (1967, 1969) has made the only previous extensive observations of *A. abdita* in its natural habitat, studying them on an intertidal sandy flat in Barnstable, MA. He found that the abundance of *A. abdita* was irregular. The substrate sediments were stable when the mats were present, but unstable when absent. The abundances of associated macroinvertebrate species fluctuated accordingly.

*A. abdita* feeds at the tube mouth collecting diatoms, probably flagellates, amorphous organic material and clay-silt grains. It digests the living and organic matter, and forms the clay-silt grains and diatom cases into pellets to be cast onto the surface substrate below them (Mills 1967, Redmond et al. 1994).

Each generation of *A. abdita* is short-lived, the males and females producing one brood of juveniles. Adults mate in the water column (Mills 1967, Borowsky & Aitken-Ander 1991) and die shortly afterward. After about 2 wk in brood pouches of the females, the juveniles are about 1.5 mm long, and they are released

from their tubes. The females then die shortly afterward. The juveniles construct a new mass of tubes (Mills 1967).

The planktonic invertebrates in Raritan Bay include copepods (mainly, *Acartia tonsa* in summer and *A. clausi* in winter), polychaete and nemertean larvae, amphipod zoeae, barnacle nauplii, mysid larvae of shrimp, cyprids, crab zoeae, gastropod veligers and fish larvae (Yamazi 1962, Jeffries 1964, Croker 1965). The plankters presumably produce large quantities of fecal pellets. Recent studies have shown that copepods break down most fecal pellets distributed through the water (Hofmann et al. 1981, Bathmann et al. 1987, Lampitt et al. 1990, Noji 1991, Noji et al. 1991, Viitasalo et al. 1999). As they descend onto the bottom, the fragmented pellets likely mix with settled phytoplankton and become a component of the sediment that accumulates with the *A. abdita* pellets in the mud habitat. At least eight fish species are present seasonally; some prey on *A. abdita* (Wilk & Silverman 1976, Collette & Klein-MacPhee 2002).

## MATERIALS AND METHODS

### Benthic Sampling

Fifty-four stations located in the mud habitat were sampled to determine the extent and the characteristics of the *A. abdita* tubes. At each station, two samples were taken with a Petite Ponar hand grab with an opening of 15 cm × 15 cm. The samples were brought aboard the vessel, the hand grab was opened, and then a shallow sample was scraped from the sediment surface into a beaker, and later viewed under a dissecting microscope, as proposed by Watling (1991). The pellets were measured and photographed using a scanning electron microscope at the EM Facilities, Nelson Biological Laboratories, Rutgers University, Piscataway, NJ. Sediment was collected from the sand areas to determine their grain sizes ( $\phi$  value). This was done by passing them through a stack of sorting screens.

The total organic carbon (TOC) in the *A. abdita* tubes was determined by collecting them at several mud stations. The TOC in the sand sediments was determined by taking cores of bottom sediments on January 17, 2003. The tubes and sediments were spooned separately into small glass jars aboard the vessel. The samples later were placed in a refrigerator overnight, and the tubes

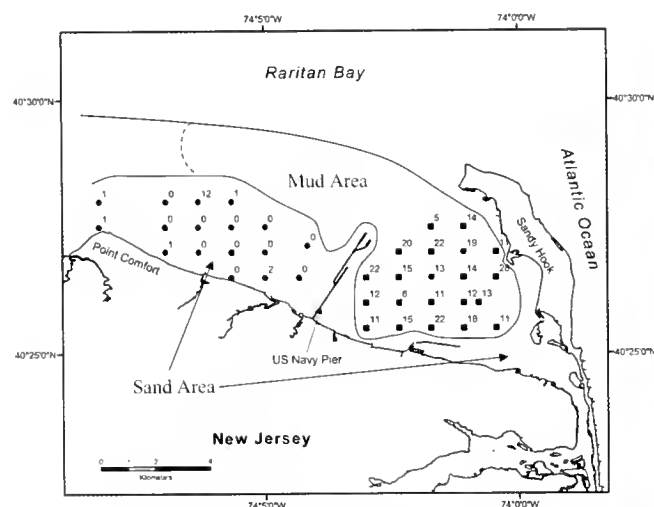


Figure 1. Abundance (number m<sup>-2</sup>) of >30 mm northern quahogs, *Mercenaria mercenaria*, in mud and sand areas in southeastern Raritan Bay (Celestino 2003). The mud area is outlined. The study's sampling area for examining the autecology and the abundance of *A. abdita* and quahog predators extended throughout the mud area to the broken line to the west. The abundance of quahog predators was surveyed in the two sand areas.



Figure 2. Section of bottom (side view) in southeastern Raritan Bay showing mass of *A. abdita* tubes. The oxidized layer directly beneath them consists of pellets. An anaerobic mud layer lies below it.

afterward were washed of loose particles under a freshwater faucet. The tubes and sand were put into separate aluminum cups and dried at 110°C for at least 24 h, weighed and then held in a muffle furnace at 510°C for 4 h and reweighed. The weight loss in the furnace was considered to be the percentage of TOC present.

The *A. abdita* tubes were sampled on 37 dates from August 22, 2001 through October 17, 2005. Most but not all stations were sampled on every survey date. Upon retrieval, the grab cover was opened and the extent of coverage of amphipod tubes, from 0% to 100%, was estimated visually. A surface with 0% coverage had no tubes, whereas a surface with 100% tubes was entirely covered with a dense mass of erect tubes (Fig. 3A). In addition, the age of the tubes at each station was recorded, as new (recently formed), middle-aged (upright but obviously not new or disintegrating), or old (disintegrating) (Fig. 3B). The contents of the grab were emptied into a 0.5-mm mesh sieve, spooned into labeled jars, and later in the day buffered formalin was added to the jars to preserve the sample. After 4 to 5 days, the formalin was replaced with 70% alcohol. The *A. abdita* and their tubes were counted using a dissecting microscope.

The relative abundance of predators was determined by sampling four fixed stations spaced across each of the mud and sand habitats with a specially designed dredge. The dredge had a mouth opening 60-cm wide. The blade at its bottom was 7-cm wide and angled at 45° so the predators in its path would slide up and into the fine-meshed bag (2-mm mesh openings) of the dredge. A planing board, 12.5-cm wide, was fastened 60 cm ahead of the mouth and positioned at the same level as the mouth to kick up predators from the bottom to collect in the bag. The dredge was towed along the bottom at a speed of 1.5 knots for 4 min at each station. When retrieved, its collected material was bagged and the predators later were identified and counted. The eight stations were sampled every 4–6 wk on 10 dates during 1999 (April 12 to October 22), on 8 dates in 2000 (January 26 to August 22), and on 2 dates in 2001 (May 9 and September 20).

### Laboratory Observations

*A. abdita* were observed constructing its tubes in laboratory pans to aid in explaining its distribution in the mud and sand sediments. Three types of sediments were tested in plastic pans, 20 × 35 cm and 13-cm deep. Each pan received a different sediment: (1) medium-sized sand; (2) a collection of mud from the surface of the mud habitat and (3) an inorganic paste consisting of fullers earth (clay) and raw seawater. To prepare the mud, it was spray-washed with seawater through a 0.63 µm sieve to break up any *A. abdita* pellets. The three sediments were spread in a layer 5 mm thick over the pan bottoms. Trays were then filled with raw seawater and air stones added. Finally, about 500 *A. abdita* were scattered across the bottom of each pan.

### RESULTS

The entire mud area was covered with dense mats of *A. abdita* tubes (Figs. 2, 3a, 3b); its sediment surface consisted of a layer of fecal pellets mixed with a relatively small mixture of silt, clay, fecal fragments, and other organic matter. The pellets were of 2 distinct sizes. The *A. abdita* pellets are 110–140 µm long (Fig. 4); the others are about 450 µm long. They are rod-shaped with rounded ends. As viewed under a dissecting microscope and a scanning electron microscope, they consisted of clay, silt and diatom shells probably held together by mucus. The *A. abdita* pellets were far more numerous than the larger pellets and they usually occupied about 90% of the space. The pellets were whole for a vertical distance of about 1 cm below the sediment surface. At distances between 1 and 2 cm below the surface, they were partially eroded (the larger pellets disintegrated more slowly than the *A. abdita* pellets), and below them the sediment consisted of anaerobic black silt. The TOC concentrations in *A. abdita* tubes were 10.8% and 13.2% in two determinations on January 17, 2003.

### *Ampelisca Abdita* Tube-pellet mud habitat

The *A. abdita* tube mats were consistently present over the entire mud area. The tubes usually covered the mud surface so

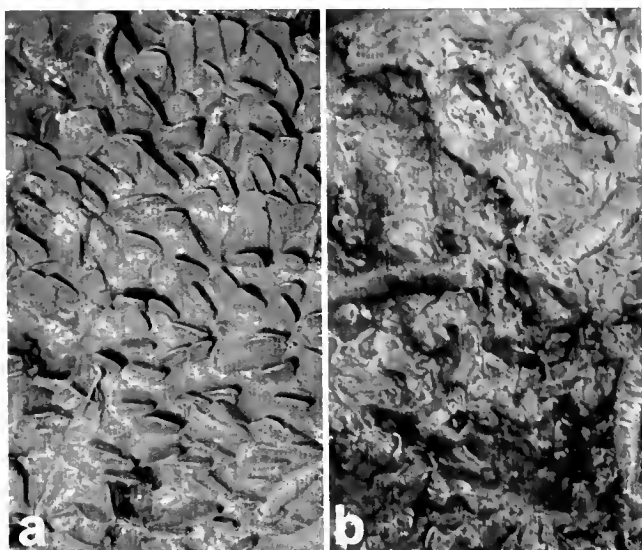


Figure 3. *Ampelisca abdita* bottom habitat. A. Mass of freshly formed *A. abdita* tubes. B. Bottom surface of decaying tubes and visible sediments; this depicts a late stage in the generation of *A. abdita* just before a new generation will settle and form a fresh mass of tubes.



Figure 4. An *Ampelisca abdita* fecal pellet viewed under a scanning electron microscope.

completely that it was not visible. This was especially true after a new formation of tubes by the juvenile *A. abdita*. Year-round sampling, which included observations of their appearances (new, middle-aged or old), showed that *A. abdita* has three breeding cycles per year. New generations settled onto the bottom and constructed new tubes in May–June, September–October and December–January. The new tubes usually were spaced densely, and so the settling of *A. abdita* juveniles must compete strongly for available space. Several weeks after the new tubes were constructed, they slowly began to disintegrate and lay flat on the bottom. At this time, the sediment surface was visible between the tubes (Fig. 3B).

The next generation of juvenile *A. abdita* and their tubes did not settle and construct its tubes over the entire 30 km<sup>2</sup> all at once, but rather large sections, as much as a third of the area was settled at a time, and then another large section was settled. The time to settle the entire area was three or more weeks.

The tube coverage of the mud habitat averaged 62% (range, 36% to 96%)/sampling day from August 2001 through October 2005 (Fig. 5). Some of the lower values were recorded during the period when the tubes were decaying. While examining the collections in the grabs, no small quahogs of any size were observed among or on the surfaces of the masses of tubes. A small number of dwarf surfclams *Mulinia lateralis* sometimes were present on the tubes and in the sediment beside them.

The numerical abundance of *A. abdita* was determined on 6 dates in 2001, 2002 and 2003 by counting their numbers in single samples from 2–8 sampling stations (34 determinations). As projected to a square meter, its density averaged 23,700 m<sup>-2</sup> (range, 989–57,185 m<sup>-2</sup>) (Table 1).

On October 23, 2001, 7 stations with 100% coverage had from 29,000–98,000 tubes m<sup>-2</sup>; a grab sample with 75% coverage had 25,000 tubes m<sup>-2</sup> and a grab sample with 45% coverage had 14,000 tubes m<sup>-2</sup>. On this date, the number of tubes present was about the same as the number of *A. abdita* present: an average of 23,290 tubes and 23,536 *A. abdita* m<sup>-2</sup> at the 7 stations.

#### Sand Habitat

The sediment in the sand habitats was medium sand ( $\phi = 1.17$ – $1.4$ ). In the broad area off the south coast of the bay, the sand surface was essentially free of any emergent structures. In contrast, the narrower sand habitat that extends westward from Sandy Hook

had scattered quahog shells covered with bryozoans and sponges and chains (stacks) of the common Atlantic slipper-snail (*Crepidula fornicata*). On January 17, 2003, the TOC was 0.29% at two areas in the sand sediments.

#### Predator Abundances

Quahog predators had a much lower abundance in the mud habitat than in the sand habitat. The longwrist hermit crab (*Pagurus longicarpus*), Atlantic oyster drill (*Urosalpinx cinerea*) and xanthid mud crabs were the most numerous quahog predators collected in our dredge. For the 20 sampling dates over three years, 1999 to 2001, their combined numbers at the four mud stations were 27 *P. longicarpus*, 11 *U. cinerea* and 175 xanthid mud crabs. The four sand stations had a combined total of 415 *P. longicarpus*, 131 *U. cinerea* and 899 mud crabs; or 15 times the number of *P. longicarpus*, 12 times the number of *U. Cinerea* and 5 times the number of mud crabs in the mud stations.

All years and stations were combined to test the main hypothesis, H<sub>0</sub>: predator density is the same in mud and sand stations. The untransformed and log-transformed sampling distributions of all three species failed normality. Mann-Whitney rank sum tests found the lower mud densities were significantly different from the higher median sand densities of all three species with  $P < 0.001$ . Mean values of predators were lower in the mud collection also, but were not tested (Table 2). The mud samples showed a lower predator density than the sand samples.

The sand habitat also had some predaceous northern moon snails (*Euspira heros*) (Greene 1978, Haskin 1951); their sand collars were found in the dredge collections in May and June each year. The dredge also collected some lady crabs (*Ovalipes ocellatus*), Atlantic rock crabs (*Cancer irroratus*) and blue crabs (*Callinectes sapidus*). The mud stations had a total of 7 of these larger crabs, whereas the sand stations had 25.

#### Laboratory Observations

In the three laboratory trays, the 500 introduced *A. abdita* dug immediately into each sediment. The *A. abdita* released in the tray with a layer of medium sand did not construct tubes.

In the tray with mud, more than 100 *A. abdita* had formed short tubes within an hour. After 56 h, the tubes were about 14-mm long, less than half the length of the tubes in the mud substrate in Raritan Bay. All were upright, and they were covered with the fine organic

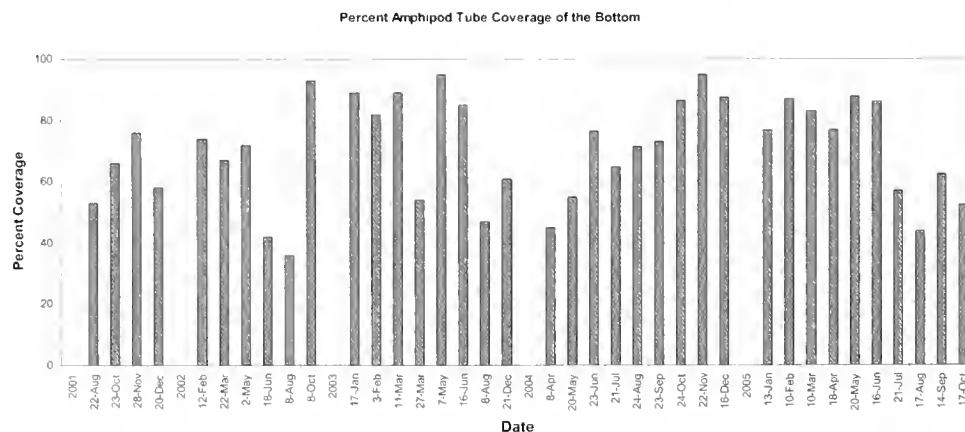


Figure 5. The percentages of the mud area covered by *Ampelisca abdita* tube mats during 2001, 2002, 2003, 2004, and 2005. The tubes were observed at every station, but the proportion of bottom covered at a particular station ranged from 0–100%.

TABLE 2.

Summary of the relative densities of the quahog predators *Pagurus longicarpus*, *Urosalpinx cinerea*, Xanthid mud crabs, and *Crangon septemspinosa* on mud and sand substrates. All years and stations are combined. Neither the untransformed nor log-transformed distributions passed normality tests. Mann-Whitney rank sum tests showed that all three species had significantly larger medians on sand at the  $P > 0.001$  level, but *Crangon* was not significantly different.

Predator	Substrate	N <sup>1</sup>	Median	Mean	Significance
<i>P. longicarpus</i>	Mud	75	0.0	0.4	<0.001
	Sand	61	4.0	11.7	
<i>U. cinerea</i>	Mud	75	0.0	0.1	<0.001
	Sand	62	0.5	2.4	
<i>Xanthids</i>	Mud	75	2.0	2.7	<0.001
	Sand	62	6.0	15.6	
<i>C. septemspinosa</i>	Mud	64	1.0	8.7	n.s.
	Sand	64	4.0	15.8	

N<sup>1</sup>—number of samples taken.

particles and silt that comprised the sediment. The particles collected on sticky mucus on the outside of the tubes as they were being formed in the midst of the sediment. The tubes did not grow further.

The *A. abdita* released in the tray whose bottom was covered with a paste of fullers earth constructed a small number of tubes—about 20 could be clearly identified. By the second day, the tubes had the overall shape of a typical tube but were much shorter. Their walls were constructed of tiny balls of fullers earth held together by mucus. Numerous fecal pellets that consisted of fullers earth were present on the sediment surface beside the tubes.

## DISCUSSION

During the 5-y field study, the entire mud area was consistently covered by dense mats of *A. abdita* tubes. In contrast, populations of *A. abdita* and their mats in the shallow sections of Boston Harbor (Gallagher & Keay 1998) and Barnstable Harbor, Massachusetts (Mills 1967), were washed away by waves produced by strong wind storms. In southeastern Raritan Bay, the *A. abdita* are able to persist during windstorms because of its deeper water (7 m avg. depth), and shorelines that protect the area from wave action. Laboratory observations suggest that *A. abdita* does not occur in habitats where sediments consist of medium and coarse sand, because they have difficulty constructing their tubes with it. *A. abdita* construct tubes readily in sediments consisting of their own pellets, silt, and also fine sand. The *A. abdita* likely are present in the mud habitat because of its grain size rather than its high organic content. The laboratory observations also suggest that juveniles begin constructing tubes as soon as they land on the bottom and the tubes are built rapidly.

The *A. abdita* tubes were free of attached macrofauna and visible plants throughout the mud habitat at all times, as Mills (1967) had observed for *A. abdita* tubes in Barnstable Harbor. The cover of the tubes' surface with their pellets and smaller particles apparently prevents macrofauna from attaching and growing. Their absence obviously favors the persistence of the *A. abdita*. Southeastern Raritan Bay has abundant macrofauna that attach to the hard surfaces of shells and stones in the sand areas and they could be present in the mud habitat if it were suitable. The species include bryozoans, blue mussels (*Mytilus edulis*), common Atlan-

tic slipper-snails, red beard sponge (*Microciona prolifera*) and the egg cases of *U. cinerea*, three lined mudsnail (*Ilyanassa trivitatta*) and eastern mudsnail (*I. obsoleta*). In other locations, some benthic invertebrates produce calcareous tubes on which other macro-invertebrates can settle and grow and eventually kill them (Russ 1980, Qian 1999, Zuhlke 2001).

It was observed that for a few weeks before each of the breeding periods, the *A. abdita* tubes gradually disintegrate, leaving a surface of pellets and fine particles available for settlement and tube building by the next generation of juvenile *A. abdita*. Can these be the periods, especially the May–June period and lesser so the September–October and least in the December–January periods, when most larvae of benthic invertebrates, including quahog pediveligers, can settle in the substrate?

It is likely that the reasons the quahogs thrive in the *A. abdita* tube-pellet mud habitat in Raritan Bay are because the water near the bottom probably has little silt, the presence of the tubes favors the settlement of their pediveligers and quahog predators are scarce and have difficulty finding small quahogs among the tubes.

Some studies suggest that mud bottoms that lack surface tubes are marginal habitats for quahogs because of the silty water associated with them (Davis 1960, Rhoads & Young 1970, Rhoads 1974). The *A. abdita* tube mats and pellets and the amphipods modify the mud surface in Raritan Bay substantially. The mats cover and stabilize it, thereby minimizing the transport of silty sediments into the water during strong currents and the movements of fish and crabs (Hunt 2005). Moreover, the mud surface consists mostly of pellets that produce little turbidity even when swirled into the water. In addition, the *A. abdita* clear the water by capturing silt and forming pellets while feeding.

The presence of *A. abdita* tubes and their pellets may provide some positive attributes for settling quahog pediveligers. The tubes serve as baffles to slow water currents, a feature that has been shown to produce higher settlements of quahog larvae (Carriker 1961, Peterson 1986, Wilson 1990). The tubes also provide a shaded zone at their bases, another feature that attracts settling quahog larvae (Carriker 1961). The settling pediveligers, that are about 225- $\mu$ m long, are more stimulated to affix their byssus to sediment grains when among small grain sizes than when among larger sizes (Carriker 2001). The *A. abdita* pellets are about half the length of quahog pediveligers whereas medium-sized sand grains are much larger than the pediveligers.

The tube mats probably are poor habitats for the predators of juvenile quahogs in Raritan Bay. All are scarce on muddy bottoms with tube mats. The long wrist hermit crab is abundant on sandy bottoms that have little structural relief and the Atlantic oyster drill, and xanthid mud crab are most abundant among oysters and shells. Where they are present among the *A. abdita* tube mats in Raritan Bay, they probably have difficulty finding the juvenile quahogs. Mills' (1967) observation that eastern mudsnails (*Ilyanassa obsoleta*) disappeared from areas that became covered with *A. abdita* tubes in Buzzards Bay is another example of gastropod numbers being low in substrates densely covered with its tubes. Similarly, earlier studies have shown that habitats containing dense upright structures, such as shells, eelgrass (*Zostera marina*) and polychaete tubes have higher abundances of quahogs and other invertebrates in their sediments (MacKenzie 1977, Beal 1983, Peterson 1986, Peterson & Beal 1989, Zuhlke 2001). The structures probably provide them cover from predators, though other factors may also play a role.

In Virginia, quahogs are equally abundant in all water depths

from 3–21 m; they appeared to be slightly scarcer at a depth of 2 m (Mann et al. 2005). This suggests that in Raritan Bay, the depth difference between the mud area, 7-m avg., and the wide sand area off the south coast, 3–4 m, and smaller sand area off the east coast, 1–2.5 m, has little influence on the difference in quahog abundance in them.

Our study has described several aspects of the quahog's habitat in mud and sand bottoms in Raritan Bay. Because we conducted a survey and not an experiment, we did not control treatment; that is mud versus sand was not randomized. Because of the contiguity of the mud and sand sites and to the hydrodynamic processes causing sediment dichotomy, there are unaccounted for differences between the sites, the effects of which may confound the findings. This situation prevails for other similar surveys and cannot be helped.

Many questions remain. Do peliveligers actually settle in higher abundances among *A. abdita* tubes? Do peliveligers attach similarly to fecal pellets and sand grains? How do infaunal invertebrates, such as nematodes and polychaetes, interact with fecal

pellets? After mature *A. abdita* females breed with males in the water, what is their behavior? How do the tubes affect predation rates on juvenile quahogs? How do finfishes prey on the *A. abdita*? Do they swallow whole tubes? What caused the starfish to abandon southeastern Raritan Bay? Can starfish inhabit bottoms covered with these tubes? How abundant were quahogs in Southeastern Raritan Bay during periods when the *A. abdita* were scarce? Will the quahogs become scarcer in Raritan Bay if the *A. abdita* become scarce again?

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## REPRODUCTIVE PATTERN OF THE SQUALID CALLISTA MEGAPITARIA SQUALIDA FROM NORTHWESTERN MEXICO

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**ABSTRACT** The annual reproductive cycle of the squalid callista *Megapitaria squalida* living in the Ojo de Liebre lagoon was analyzed from January to December 2001. The reproductive activity was analyzed qualitatively (through histological analysis), defining five gonad development stages (undifferentiated, developing, ripe, spawning and spent). A quantitative analysis was also conducted (through the gonadic index and oocyte diameter) to correlate the reproductive activity with water temperature and salinity. Male and female gonads developed and spawned in parallel through time. *M. squalida* displays a clearly seasonal reproductive cycle in the Ojo de Liebre lagoon, with a long reproductive activity period (January to August) that coincides with the rise in temperature, plus a well defined inactivity period (September to December) with 100% of undifferentiated or spent organisms and the onset of which coincides with the temperature decrease. *M. squalida*'s reproductive cycle does not seem to be influenced by salinity variations in the Ojo de Liebre lagoon. The spawning size of *M. squalida* was determined at 92 mm SL at an approximate age of 2.2 y. However, some individuals started spawning at 64 mm SL. Marked differences were found in some aspects of the reproductive pattern of *M. squalida* compared with studies conducted in other sites, and the likely causes are discussed here.

**KEYWORDS:** reproduction, hivalve, *Megapitaria squalida*, histology, Mexican Pacific.

### INTRODUCTION

The squalid callista *Megapitaria squalida* (Sowerby, 1835) is one of the most abundant bivalves in northwestern Mexico. It is distributed from the Ojo de Liebre lagoon in Baja California Sur, (including the Gulf of California) to Macora in Perú (Keen 1971). *M. squalida* lives in muddy and sandy bottoms at depths ranging from one to 120 m, and can reach up to 120 mm in body length (Singh et al. 1991).

Studies on growth (Castro-Ortiz et al. 1992), ecological aspects (Baquero & Stuardo 1977, Anguas-Vélez & Castro-Ortiz 1990, Singh et al. 1991), mantle anatomy (García-Gasca & García-Domínguez 1995), and reproductive aspects (Baquero & Stuardo 1977, Singh et al. 1991, Villalejo-Fuerte et al. 1996a, Baquero & Aldana 2000, Villalejo-Fuerte et al. 2000) have been conducted in *M. squalida*.

Although *M. squalida* is usually considered as a species with a low commercial value, in northwestern Mexico the fishery of this clam has intensified recently, resulting in an increase in production from 31.4 tons in 1996 to about 972 tons in 2004 (SAGARPA 2006). The above derives from the fact that the species is being captured, more and more, as an alternative resource when the main commercial species are unavailable because of the enforced fishing restrictions. *M. squalida* is captured throughout the year from the main water bodies along the state of Baja California Sur, including the Gulf of California and the Mexican coasts of Pacific Ocean. The capture regimen of *M. squalida* is based on previous studies of some populations from the Gulf of California (Singh et al. 1991, Villalejo-Fuerte et al. 1996a, 2000); however, the reproductive cycle varies with the geographic location, according to the species' specific phenotypic response to the particular environmental conditions in each location (mainly water temperature and food availability) (Porter 1964, Hesselman et al. 1989, Cruz & Villalobos 1993, Rodríguez et al. 1993).

Hence, a basic understanding of the reproductive cycle of *M. squalida* is needed for an appropriate timing of the fishing effort and the development of a sustainable fishery. Thus, the objective of this study is to determine the reproductive cycle of *M. squalida* in the Ojo de Liebre lagoon, located in the Pacific coast, and compare it with studies conducted in other localities.

### MATERIALS AND METHODS

The Ojo de Liebre lagoon (24°02'N, 110°24'W) is located within the Sebastián Vizcaíno bay, in the west coast of the Baja California peninsula, Mexico (Fig. 1). There, 30 *M. squalida* adult specimens were collected per month by a scuba diver at 3- to 6-m deep from January to December 2001. The bottom water temperature and salinity were recorded at the time of sampling by means of a U-10 HORIBA water-quality analyzer. In the laboratory, total and soft body weights and shell length (SL) were recorded for each clam. To determine the extent of gonad development, the visceral mass of each clam (gonad included) was dissected and fixed in 10% formalin. Later, a section of tissue from the dorsal area of each visceral mass was dehydrated in alcohol and embedded in paraplast X-tra. Sections (7 µm) were placed on slides and stained with haematoxylin and counterstained with eosin (Humason 1979).

Because *M. squalida* does not exhibit sex dimorphism, specimens were sexed through histological analysis. Sex ratios were analyzed by month, testing the null hypothesis of a 1:1 sex ratio, using a chi-square test ( $\alpha = 0.05$ ) (Zar 1996).

Qualitative characteristics were used to sort specimens into one of five arbitrary gonad stages previously established for the same species: undifferentiated, developing, ripe, spawning and spent (Baquero & Stuardo 1977, Villalejo-Fuerte et al. 2000). The relative frequencies of gonad-development stages throughout the year were obtained. This enabled the description of the reproductive cycle.

To obtain quantitative information on the reproductive activity, the oocyte diameter and a monthly gonad index were determined. Oocyte diameter was measured from digitalized images of histo-

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Figure 1. Localization of the areas in the Mexican Pacific coast where the reproduction of *M. squalida* has been studied. (1) Ojo de Liebre lagoon, (2) Concepción bay, (3) Juncalito bay, (4) La Paz bay, (5) Zihuatanejo bay.

logical sections by means of the SCAN PRO software (Version 5.0, Systat Software, Inc., Point Richmond, CA, USA). Oocytes were individually traced by hand with the pointer, and the software automatically measured the major- and minor-axis lengths. Then, both dimensions were averaged and this was considered as the estimated diameter. In this way, a monthly mean oocyte diameter was calculated. At least 100 oocytes were measured from each slide. Only oocytes showing a nucleolus were sampled, assuming that this structure is located at the cell center (Laruelle et al. 1994). Individuals with few measurable oocytes and extensive phagocytosis (i.e., "spent" specimens) were excluded following the criteria of Grant and Tyler (1983). A one-way ANOVA, followed by a Tukey posthoc mean comparison test, was used to assess significant differences between months. The monthly gonad index was computed using a numerical grading system (Heffernan et al. 1989). Four categories were established according to the degree of gonad development, with 0 = undifferentiated and spent, 1 = developing, 2 = partially spawned and 3 = ripe. The monthly gonad index was determined by multiplying the number of specimens assigned to each category by the category score, summing all such values and dividing this amount by the total number of clams analyzed. The monthly values of oocyte diameter and gonad index obtained, enabled to carry out correlation analyses of reproductive activity with temperature and salinity, using a Spearman-rank correlation analysis (Zar 1996). The STATISTICA (version 6.0) software (StatSoft, Inc. 2001) was used for all statistical analyses. The significance level ( $\alpha$ ) was set at 0.05.

The size at spawning in the population is defined as the smallest length at which the 50% of the cumulative frequency of sampled females and males are spawning (Somerton 1980). To establish the size at spawning in the *M. squalida* population, the

relative cumulative frequency of ripe and spawning organisms was fitted to a logistic model. Additionally, to have a gross estimation of the age at spawning in the population, the Bertalanffy growth equation ( $L_t = 92[1 - e^{-0.545(t - 0.07)}]$ ) obtained for *M. squalida* by Castro-Ortiz et al. (1992) was used. However, for this study the  $L_\infty$  value (92) was replaced by a  $L_\infty$  value (128) calculated with the FISAT software (Gayanilo et al. 1995) using length frequencies of individuals collected in the Ojo de Liebre lagoon.

## RESULTS

A total of 384 specimens were collected, including 128 females (33.3%), 156 males (40.6%), and 100 (26%) undifferentiated specimens. The monthly sex-ratio analysis shows that a significant difference ( $P < 0.05$ ) from the expected 1:1 ratio occurred in August, September and October (Table 1). However, the sex ratio for the total sample (0.83F:1M) did not differ significantly ( $P > 0.05$ ) from the expected 1:1 ratio. Clams ranged in SL from 64 mm to 121 mm (mean = 91.3 mm, standard deviation = 9.5 mm).

The reproductive cycle of *M. squalida* in Ojo de Liebre showed a clear seasonal pattern (Fig. 2). The relative frequencies of gonad development stages throughout the year were obtained pooling females and males together, because no differences in the temporality pattern between sexes were observed. The development stage occurred from January to March, with a peak frequency in January (92.8%). Ripe clams were observed from February to August with a variable frequency. The spawning stage was observed from February to August, reaching the peak frequency in April, June, July and August (78% to 94%). From September to December 100% clams were inactive (undifferentiated and spent stages).

Oocyte diameter showed significant differences (one-way ANOVA,  $P < 0.01$ ) throughout the year (Fig. 3a). Small oocytes (30.6–33.4  $\mu\text{m}$ ) were observed from January to March. Oocyte diameter increased considerably from April to July and August, when significantly larger oocytes (44.2–45.2  $\mu\text{m}$ ;  $P < 0.01$ ) were present. The above was consistent with histological observations, which revealed that the highest reproductive activity took place from April to August. From September to December all clams were spent or undifferentiated, so that oocyte diameter values could not be obtained.

TABLE 1.

Female and male frequencies, Chi square ( $\chi^2$ ) values and sex ratios of *Megapitaria squalida* by month. Numbers in bold indicate a statistically significant differences from a 1:1 ratio ( $P \leq 0.05$ ).

Month	Female	Male	Total	$\chi^2$	Sex Ratio F:M
January	13	9	22	0.73	1.44:1
February	15	17	32	0.13	0.88:1
March	9	14	23	1.09	0.64:1
April	17	12	29	0.86	1.42:1
May	18	11	29	1.69	1.64:1
Jun	15	13	28	0.14	1.15:1
July	13	16	29	0.31	0.81:1
August	10	21	31	<b>3.90</b>	0.48:1
September	0	9	9	<b>9.00</b>	0.11:1
October	1	8	9	<b>5.44</b>	0.13:1
November	1	5	6	2.67	0.20:1
December	3	3	6	0.00	1.00:1
TOTAL	115	138	253	2.09	0.83:1

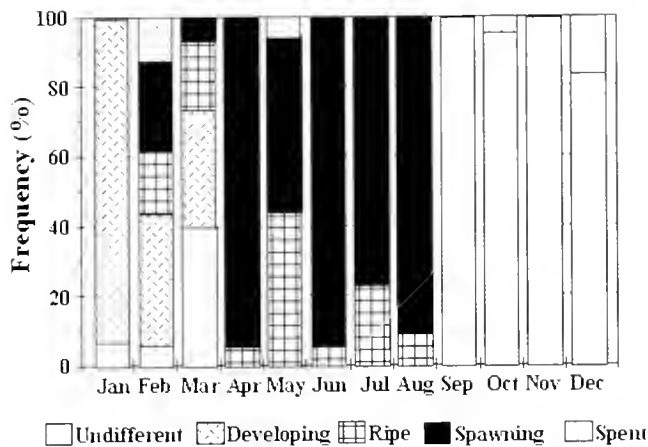


Figure 2. Monthly percent frequencies of the different gonad stages of *M. squalida* throughout the study period in Ojo de Liebre lagoon, B.C.S. México.

Gonad index displayed a seasonal tendency throughout the year (Fig. 3b). From these data, a clear resting period from September to December was also apparent, whereas gonad index values were higher from April to August, coinciding with the ripe and spawning stages.

Seawater temperature showed a clearly seasonal variation in the Ojo de Liebre lagoon (Fig. 3c). The cold months (January to April and December) had temperatures under 18°C. The lowest temperatures were registered in January and February (15.9°C). The warmer months (July to October) had temperatures above 22°C. Water temperature reached its peak value (23.4°C) in August. Salinity in the Ojo de Liebre lagoon displayed a slight fluctuation (Fig. 3d). The minimum salinity occurred in January (33.4‰), and the maximum in March and May (34.3‰). The mean oocyte diameter and gonad index were positively correlated with temperature ( $R = 0.86$ ;  $P = 0.006$  and  $R = 0.76$ ;  $P = 0.03$ , respectively), but not with salinity ( $R = 0.24$ ;  $P = 0.57$  and  $R = 0.54$ ;  $P = 0.17$ , respectively).

To establish the size at spawning in the *M. squalida* population studied, relative cumulative frequency data of ripe and spawning organisms were fitted to the logistic model ( $r = 0.99$ , Fig. 4). The size at spawning in the *M. squalida* population was 92 mm SL, which may correspond to an age of 2.2 y old (according to our gross estimation). However, individual organisms may start spawning at 64 mm SL (1.2 y old).

#### DISCUSSION

Slight differences in seasonal gametogenesis and spawning patterns between males and females have been reported in some bivalves (Goodwin 1976, Sloan & Robinson 1984, Baron & Cioeco 2001). In our study, male and female gonads developed and underwent spawning in parallel through time, as reported in other bivalves (Villalejo-Fuerte et al. 2002, Choi & Chang 2003). This synchronization seems to be related to changes in water temperature, as reported by Gribben et al. (2004) for *P. zelandica*, and it is characteristic of species living in temperate zones.

Many studies point out that temperature is the most important environmental factor in the regulation of reproduction in bivalves (gametogenesis and spawning) (Giese & Pearse 1974, Sastry 1979, Barber & Blake 1981, Cruz & Villalobos 1993, Rodríguez et al. 1993, Wada et al. 1995), which may trigger or synchronize the

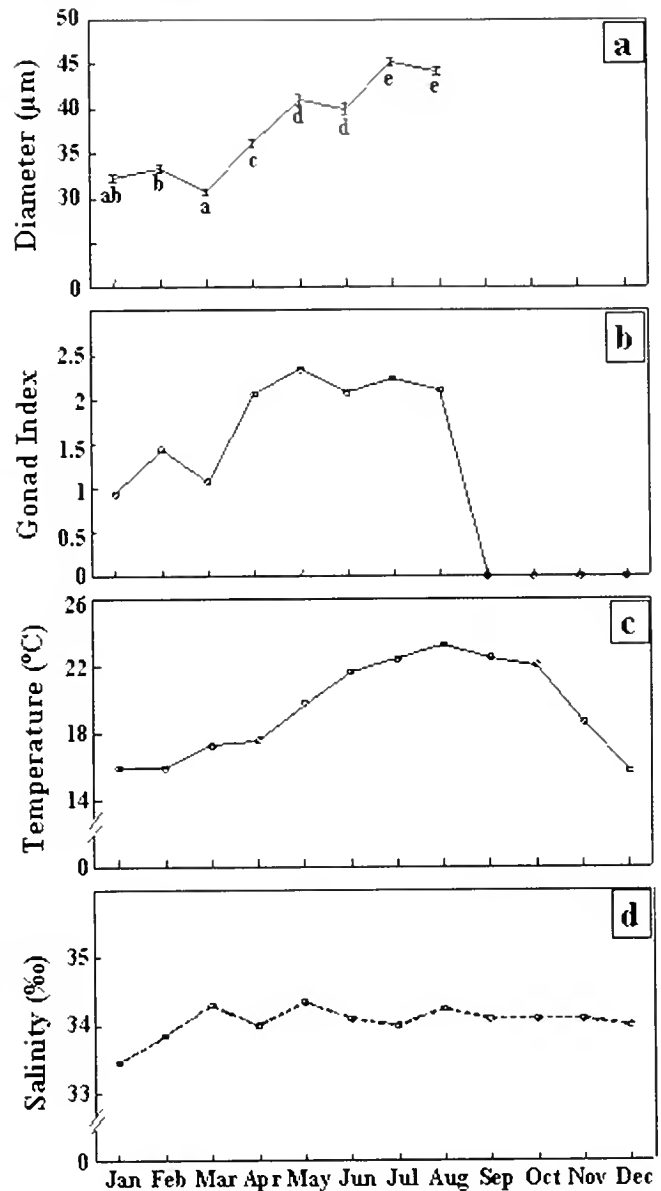


Figure 3. Temporal variation of the different variables analyzed throughout the year in the Ojo de Liebre lagoon. (a) Oocyte diameter, (b) gonad index, (c) bottom seawater temperature, (d) salinity. Oocyte diameter was analyzed by one-way ANOVA followed by Tukey test. Means not sharing the same superscript are significantly different. Bars correspond to standard errors.

“temporality” of the different development stages (Lubet 1983, Gallardo 1989). Thus, the rise in water temperature may stimulate gonad ripening (Giguere et al. 1994). In Ojo de Liebre, the reproductive activity of *M. squalida* (measured through the variation in the mean oocyte diameter and gonad index) was significantly and positively correlated with water temperature. The relationship between temperature and reproductive activity is well documented for other bivalves living in the Mexican Pacific coasts, including *Chione californiensis* (García-Domínguez et al. 1993), *Glycymeris gigantea* (Villalejo-Fuerte et al. 1995) and *Laevicardium elatum* (Villalejo-Fuerte et al. 1996b).

In Ojo de Liebre lagoon, *M. squalida* shows a clearly seasonal reproductive cycle. This includes an extended period of reproduc-

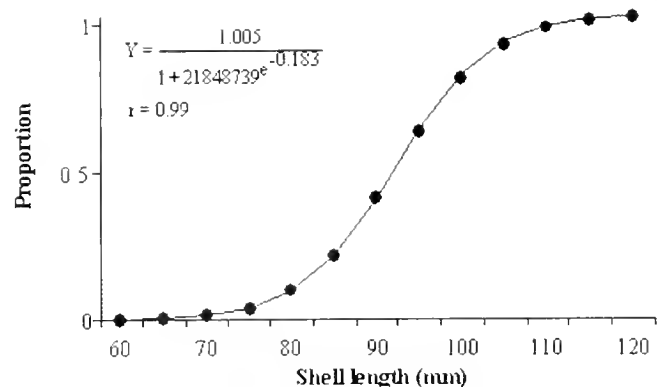


Figure 4. Length of spawning of *Megapitaria squalida* from Ojo de Liebre lagoon, B.C.S., México.

tive activity (January to August) coinciding with the period of temperature increase, followed by a well-defined reproductive inactivity (September to December) where 100% of organisms are either undifferentiated or spent, the onset of which coincided with the drop of temperature. Likewise, a seasonal reproductive pattern has already been reported in the same location for other bivalves such as *Nodipecten subnodosus* (Arellano-Martínez et al. 2004a).

However, although temperature undoubtedly affects reproduction, other environmental factors may seem to interact with it to determine the annual reproductive activity pattern in any given geographic area (Sastri 1970). In this sense, the variation in the onset of gonad proliferation in *Ruditapes decussatus* has been found to be caused by interannual salinity fluctuations (Shafee & Daoudi 1991), and spawning in *A. ventricosus* coincides with minimum and maximum salinity values (Luna-González et al. 2000). In other cases, extreme salinity values have been found to lead to a steady reduction in the duration of gonad development (Muranaka & Lannan 1984, Utting 1993). To this respect, salinity shows hypersaline gradients in the Ojo de Liebre lagoon (Lankford 1977), with salinities over 47 ‰ recorded in some areas (Contreras, 1985), being either shallow zones or as a result of human activities (brine discharges by the salt-producing company into some areas of the lagoon). However, in this investigation salinity in the study area (El Borbollón) was found to be relatively constant throughout the whole study period, likely caused by its closeness to the lagoon mouth. Thus, the reproductive cycle of *M. squalida* in this locality seems to be unaffected by salinity variations, be-

cause no significant correlation was found with this variable. This does not rule out the possibility that another clam bank located farther into the lagoon (where greater salinity variations occur) might be affected by this environmental factor. However, further research is required to obtain concluding results to this respect.

Currently, there is available information on reproductive aspects of *M. squalida* from four additional localities of the Mexican Pacific coast (Fig. 1): in the Gulf of California, from Bahía Concepción (Villalejo-Fuerte et al. 1996a), Bahía Juncalito (Villalejo-Fuerte et al. 2000) and Bahía de La Paz (Singh et al. 1991), and outside the Gulf of California, from Bahía Zihuatanejo (Baquero & Stuardo 1977), a site located in the southern Mexican Pacific coast. Our findings reported here differ substantially in some aspects with the studies mentioned earlier (Table 2). The most obvious difference refers to the temporality of the reproductive cycle, which might be associated with water temperature and/or food availability in each locality.

As mentioned earlier, in Ojo de Liebre lagoon *M. squalida* displays a marked interruption in the reproductive activity (high percentage of undifferentiated or spent organisms) from September to December. However, this complete interruption of the reproductive activity during the annual cycle was absent in any other locality, whereas the undifferentiated stage occurred only at low levels in some months (Singh et al. 1991, Villalejo-Fuerte et al. 1996a, Baquero & Stuardo 1977, Villalejo-Fuerte et al. 2000). This clear resting period at the Ojo de Liebre lagoon might derive from having included immature specimens. However, the range of sizes analyzed (64 mm to 121 mm SL) is larger than the size of the smallest specimen (42 mm SL) reported as having undergone spawning (Villalejo-Fuerte et al. 1996a), so that this finding is valid. In this sense, Baquero and Aldana (2000) point out that the undifferentiation or lack of reproductive activity periods is not species-specific requirements, but responses to environmental conditions. Villalejo-Fuerte et al. (2000) mention that the absence of a reproductive inactivity period in Bahía Juncalito may result from food being abundant throughout the year, so that the clam population produces gametes continuously. This situation might also occur in the other localities of the Gulf of California, as well as in Bahía Zihuatanejo. In contrast, the Ojo de Liebre lagoon has been regarded as an area with low food availability (Millán et al. 1987, Delgadillo-Hinojosa et al. 2002, Arellano-Martínez et al. 2004b) and low food quality (Arellano-Martínez et al. 2004b). This situation of low food availability and quality in Ojo de Liebre might lead to the marked resting period and foster a

TABLE 2.

Available information on reproductive aspects of *Megapitaria squalida*.

Locality	Sex Ratio F:M	Oocyte Diameter	Resting Period	Spawning Period (Main Period)	Source
Zihuatanejo Bay	0.88:1	—	Not defined	All months except July (October to May)	Baquero & Stuardo 1977 Baquero & Aldana 2000
La Paz Bay	—	—	Not defined	All months (August to October)	Singh et al. 1991
Juncalito Bay	1.02:1	25–40 µm	Not defined	All months (July to August)	Villalejo-Fuerte et al. 2000 Quiñones-Arreola 2003
Concepción Bay	1:1	—	Not defined	All months (June to August)	Villalejo-Fuerte et al. 1996b
Ojo de Liebre lagoon	0.83:1	30.6–45.2 µm	September to December	February to August	This work

marked energy storage/use cycle, as reported by Arellano-Martínez et al. (2004b) for *N. subnodosus* in the same locality. Nevertheless, further studies are required on this aspect to corroborate it for *M. squalida*.

On the other hand, in the case of the populations living in the Gulf of California and Bahía de Zihuatanejo, the ripe and spawning stages were present (although at different times and proportions) throughout the whole study period (Table 2), so that it is assumed that *M. squalida* from these localities displays a continuous reproductive cycle. This reproductive behavior has also been observed in other bivalves from tropical latitudes, including *Laevicardium elatum* (Villalejo-Fuerte et al. 1996b), *Periglypta multicostrata* (García-Domínguez et al. 1998), *Pinna rugosa* (Ceballos-Vázquez et al. 2000) and *Spondylus calcifer* (Villalejo-Fuerte et al. 2002). In contrast with the above, in the Ojo de Liebre population the ripe and spawning stages are restricted to the period January to August, which indicates that the reproductive cycle of *M. squalida* in this locality seems to be seasonal. This lagoon is considered as a temperate geographic zone (Briggs 1974), whereas the Gulf of California is regarded as a tropical-subtropical transition zone (Hendrickx et al. 2005) and Bahía de Zihuatanejo, a tropical one (Briggs 1974). Differences in the reproductive pattern of any given species are known to occur along a latitudinal range, given that critical temperatures (cold or warm) occur in different months or prevail all the year round, facilitating a continuous reproduction (Hesselman et al. 1989). The earlier mentioned coincides with reports by Porter (1964) and Hesselman et al. (1989), who mention that the differences in the reproductive cycle among various populations seem to result from phenotypical responses to variations in environmental conditions, mainly food availability and temperature. To this respect, Baqueiro and Aldana (2000) state that some mollusc species display different gonad-recovery and spawning patterns associated with the microenvironmental conditions of their habitat.

Furthermore, this study revealed that the mean oocyte diameter of *M. squalida* living in the Ojo de Liebre lagoon was larger (30.6–45.2  $\mu\text{m}$ ) than the one reported by Quiñones-Arreola (2003) for *M. squalida* living in Bahía Juncalito, Gulf of California (25–40  $\mu\text{m}$ ). Oocyte growth depends on an adequate energy supply available for gametogenesis (Barber & Blake 1983). The availability of enough energy depends on both, food availability and the animal's metabolic rate, which to some extent is influenced by environmental temperature (Barber & Blake 1983). As mentioned earlier, food availability is high all the year round in Bahía Jun-

calito, which should favor a larger oocyte size, something that does not occur. This could be explained assuming that the metabolic rate in Bahía Juncalito might be higher than in Ojo de Liebre, given that temperature in the former is 5 °C higher (Quiñones-Arreola 2003). Additionally, the constant oocyte production (continuous reproductive cycle) in Bahía Juncalito involves more accelerated periods of gonad recovery and, consequently, smaller oocytes compared with oocytes from Ojo de Liebre are produced. For its part, food is not abundant in the Ojo de Liebre lagoon but there is a marked resting period, which would favor nutrient accumulation. Afterwards, nutrients would be available for gonad ripening, favoring a larger oocyte size.

This study found that the sex ratio in the population studied was 1:1. This finding coincides with reports for this same species in other localities (Table 2). This indicates that sex ratio is a species-specific characteristic unaffected by environmental conditions. Villalejo-Fuerte et al. (2000) infer, from a 1:1 sex ratio, that mortality in *M. squalida* (either natural or by fishing) does not differ between sexes, that no sexual dimorphism occurs in terms of size (because clams are caught only in the case of large specimens), and that growth rate seems to be similar for males and females. Nonetheless, further research is required to confirm the above.

As regards the size at spawning of *M. squalida* (92 mm SL) no comparisons are possible, given that no background information is available. However, smaller spawning organisms (42–50 mm SL) were reported in Bahía Concepción (Villalejo-Fuerte et al. 1996a), which are even below the smallest size reported in this study for a spawning organism (64 mm SL). According to the earlier mentioned, the size at spawning in Ojo de Liebre might be overestimated in this study. To corroborate this hypothesis, further research is required including smaller organisms.

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## RESTRICTION ENZYME DIGESTION CHROMOSOME BANDING ON TWO COMMERCIALY IMPORTANT VENERID BIVALVE SPECIES: *RUDITAPES DECUSSATUS* AND *CERASTODERMA EDULE*

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**ABSTRACT** Reliable banding techniques are a major necessity for the genetic research in marine bivalves. Restriction enzyme banding (HaeIII) was performed, in this study, on chromosomes of two commercially important species of veneroid bivalves: the clam *Ruditapes decussatus* (Adams and Reeve) and the cockle *Cerastoderma edule*. Identification of the nineteen individual chromosome pairs was obtained for both species. The cytogenetic studies made in marine molluscs have recently experienced a very fast development caused by the introduction of new molecular techniques mainly fluorescence *in situ* hybridization (FISH). Recently it has been shown in mammalian chromosomes that restriction enzyme banding is compatible with FISH, allowing simultaneous banding, and consequent accurate identification of the localization of the probes and unambiguously identification of the chromosome(s) carrier(s). As far as we know this is the first RE-banding obtained in karyotypes of veneroid species. The application of restriction enzyme chromosome banding in veneroids are diverse and this study can constitute a fundamental step for future gene mapping on this commercially important group of bivalves and could offer a new approach to specific problems in veneroid taxonomy and genetics.

**KEY WORDS:** *Cerastoderma edule*, chromosome banding, *in situ* restriction enzyme banding, *Ruditapes decussatus*, veneroid

### INTRODUCTION

Cytogenetic investigations in veneroid and marine bivalves in general were first mainly concerned with data on chromosome number and gross morphology. Later, morphometric analysis of karyotypes provided characterization of chromosome morphology based on centromeric position. Afterwards, the application of differential staining techniques such as Ag-NORs for nucleolar organizer regions, C-banding for heterochromatin or G-banding for individual chromosome identification allowed the identification of specific chromosome pairs in the karyotypes of bivalve species (see Thiriou-Quiévreux 2002, for review).

In the last 20 years, the introduction of new molecular techniques essentially fluorescence *in situ* hybridization (FISH) allowed a significant development in the cytogenetic studies made in marine molluscs. However the classical banding techniques such as G-, R- or Q-banding used for individual chromosomal identification, are not compatible with FISH. In fact these bandings are often lost during the *in situ* hybridization procedure even following refixation using relatively low temperatures and short times for denaturation (Chaves et al. 2002) making difficult the accurate chromosomal localization of the probes and the identification of the chromosome(s) carrier(s).

In several recent studies on the application of the FISH technique to marine bivalves, and although these applications were successful in obtaining positive signal(s) of hybridization, a difficulty in the unambiguous identification of the exact chromosomes carrier of the probes was encountered, except on the cases of localization of the probes on the largest or smallest chromosome pairs, which can be easily distinguished by their highly differentiated size. Chromosome carriers could only be barely identified based on their size and centromeric index, not by means of individual chromosome banding identification (e.g., Clabby et al. 1996,

Zhang et al. 1999, Insua et al. 1999, Gonzalez-Tizon et al. 2000, Xu et al. 2001, Wang & Guo. 2004, Hurtado & Pasantes 2005), which unfortunately limits the extent of the potential of this technique.

*In situ* digestion with restriction endonucleases (REs), which cleave DNA at specific target sequences, has been shown to produce consistent banding patterns in fixed mammalian and insect chromosomes and more recently has been successfully applied to mussels (Martínez-Lage et al. 1994), scallops (Gajardo et al. 2002) and oysters (Leitão et al. 2004, Bouilly et al. 2005, Cross et al. 2005). In all cases, specific longitudinal chromosomal banding patterns were obtained after digestion with REs, allowing the individual identification of all chromosome pairs and the establishment of precise karyotypes. This technique has also been applied in a chromosomal evolution study within the Ostreidae family (Leitão et al. 2004).

RE banding presents a major advantage, in fact it has been recently shown in mammals that restriction enzyme banding is compatible with FISH (Chaves et al. 2002). Moreover, the combined use of different REs can also be useful in the detection of different classes of heterochromatin not revealed by standard banding techniques.

Genetic studies of commercially important marine bivalves have considerably increased in recent years, becoming crucial for the development in aquaculture. The harvesting of veneroid bivalve species has been an important component of European and Northern Africa fisheries since ancient times. The clam *Ruditapes decussatus* and the cockle *Cerastoderma edule* are of great socio-economic importance in Europe and are widely distributed along the coastline. The culture of these bivalve molluscs, mainly the culture of the clam, *R. decussatus*, represents a major fraction of the molluscan mariculture in Portugal with over 10,000 people directly or indirectly involved in this activity (Ruano 1997, Ruano & Cachola 1986).

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Métivier 1971, and Partridge, 1977 for taxonomic revision) are of considerable commercial importance but very little has been published on their basic genetics. Only 2 (Wilkins & Mathers 1974, Walne & Wood 1975) of the 225 references cited by Partridge (1977) on *R. decussatus*, concern genetics. Since 1975, genetic studies on the genus *Ruditapes* remain scarce (Borsa & Thiriot-Quiévreux 1990). In cytogenetics concerns, the studies consisted only of chromosome number determination of *R. phillipinarum* and *R. decussatus* ( $2n = 38$ ) (Gérard 1978), triploidy induction in *R. phillipinarum* (Beaumont & Contaris 1988, Goslin & Nolan 1989), and only standard karyotype characterization with intragenus comparison of *R. phillipinarum*, *R. aureus* and *R. decussatus* (Borsa & Thiriot-Quiévreux 1990).

Very little karyological data have been published until now on *Cerastoderma*. A chromosome complement of  $2n = 38$  has been reported in *C. edule* and *C. glaucum* (Koulman & Wolff 1977). Only standard karyotype has been described in an Atlantic population of *C. edule* (Insua & Thiriot-Quiévreux 1992) and standard karyotype, and the location of the nucleolar organizer regions were described from Baltic and Mediterranean populations of *C. glaucum* (Thiriot-Quiévreux & Wolowicz 1996). FISH was successfully applied in *C. edule* for the study of the 5S rDNA repeated unit (Insua et al. 1999).

However, up till now, no banding technique, which allows the individual identification of all chromosome pairs was applied to any of these species. The unambiguous identification of all individual chromosome pairs is essential for: (1) the establishment of the precise karyotype of these species; (2) chromosome evolution studies (through the study of possible fissions, translocations, and deletions); (3) aneuploidy studies and (4) the precise localization of *in situ* hybridization probes, because the chromosomes pairs carrying the probes cannot be accurately determined without unambiguous identification of all chromosome pairs.

To fulfill this gap in these two veneroid species, we applied in this study the restriction enzyme banding technique to fixed metaphase chromosomes of *R. decussatus* (Veneridae) and *C. edule* (Cardiidae).

## MATERIALS AND METHODS

### Biological Material

Specimens of *R. decussatus* were intertidally collected at Lameirão (Ria Formosa lagoon, south of Portugal) and specimens of *C. edule* were intertidally collected at Almargem (Ria Formosa lagoon, south of Portugal). Before processing, the animals of both species were acclimated at the IPIMAR-Culture Molluscs Experimental Station Hatchery for one week.

### Chromosome Preparation

Whole juvenile animals (ca. 1.5-cm length) were incubated for 7 h in a 0.005% solution of colchicine in seawater. Then the gills were dissected and treated for 30 min in 0.9% sodium citrate in distilled water. The material was fixed in a freshly prepared mixture of absolute alcohol and acetic acid (3:1) with three changes of 20 min each. Fixed pieces of gill from each individual were dissociated in 50% acetic acid with distilled water solution. Slides were prepared following an air-drying technique (Thiriot-Quiévreux & Ayraud 1982). The slides were kept at  $-20^{\circ}\text{C}$  until further used.

### In situ Restriction Endonuclease Digestion

Slides were aged during 6 h, in a dry incubator at  $65^{\circ}\text{C}$ , before the restriction endonuclease treatment. The restriction enzyme used: HaeIII (GG/CC) was diluted in the buffer indicated by the manufacturer (Invitrogen, Life Technologies), and final concentrations of 30 U were obtained per 100  $\mu\text{L}$  (following Leitão et al. 2004). The 100- $\mu\text{L}$  of this solution was placed on each slide and covered with coverslips. These slides were incubated in a humid chamber for 16 h at  $37^{\circ}\text{C}$ . Control slides were submitted to the same treatment as described earlier in this study but incubated only with buffer. The slides were then washed in distilled water, air dried and stained with Giemsa (1% solution, diluted in phosphate buffer at pH 6.8).

### Microscopy and Image Processing

Images of metaphases of *R. decussatus* and *C. edule* banded with the restriction endonuclease HaeIII were acquired with a CCD camera (AxioCam, ZEISS) coupled to a ZEISS Axioplan 2 Imaging microscope. Digitized photos were printed from Adobe Photoshop (version 5.0) using only contrast optimization functions that affected the whole of the image.

### Karyotype Organization

The karyotypes of the banded metaphases were organized based on the length, centromeric position and RE-banding pattern. Because we are working with somatic tissues, we had to use many animals to obtain a sufficient number of mitoses. A total of 38 RE-banded karyotypes were examined for *R. decussatus* and 42 for *C. edule*.

## RESULTS

The diploid complement of both *R. decussatus* and *C. edule* was  $2n = 38$ , and the proportion of the different morphometric types of chromosomes observed in both species was similar to the one observed in previously published results, 6 metacentric, 3 submetacentric and 10 subtelocentric pairs for *R. decussatus* (Borsa & Thiriot-Quiévreux 1990) and 12 submetacentric, 4 subtelocentric and 3 telocentric for *C. edule* (Insua & Thiriot-Quiévreux 1992).

The RE (HaeIII) tested yield specific banding pattern in the 38 RE-banded karyotypes examined of *R. decussatus* and the 42 of *C. edule*. Moreover, the banding patterns were consistent between members of homologous chromosome pairs. The *in situ* RE experiment was compared with control treatment on slides from both species. Control slides were tested with the same treatment as the *in situ* restriction banding slides, but incubated only with buffer. In both cases, there was no banding pattern induced in the chromosomes, and all chromosomes incubated (from both species), with only buffer from HaeIII showed a Giemsa standard staining.

Examples of banded metaphases with HaeIII are present in Figure 1 for *R. decussatus* (Fig. 1a) and *C. edule* (Fig. 1b). Karyotypes with consistent banding pattern between homologous pairs are shown in Figure 2 for *R. decussatus* (Fig. 2a) and *C. edule* (Fig. 2b). All results are assembled and summarized in Figures 3 and 4, which show the haploid distribution of HaeIII chromosomal bands in the two species. HaeIII produced a banding pattern along the length of each chromosome (Figs. 3 and 4). The restriction banding produced was adequate for the single identification of all chromosomes for both species and organization of their respective



Figure 1. Examples of banded metaphases with the RE HaeIII: Figure 1a, metaphase of *R. decussatus*; Figure 1b, metaphase of *C. edule*. Scale bar = 5  $\mu$ m

karyotypes (Figs. 2, 3 and 4). Interstitial, centromeric and telomeric bands were observed along the chromosomes of both species.

In the right side of each column of Figures 3 (for *R. decussatus*) and 4 (for *C. edule*) is shown a schematic representation of the *in situ* restriction banding patterns obtained for each species.

#### DISCUSSION

The diploid chromosome number of  $2n = 38$  is confirmed in both *R. decussatus* and *C. edule* and appears to be the modal number of the Veneridae and Cardiidae families (Nakamura 1985, Corni & Trentini 1986), and it is also common among the super-order Veneroida (Thiriot-Quievreux et al. 1987).

The application, for the first time, of the RE HaeIII to the chromosomes of the clam *R. decussatus* and the cockle *C. edule* produced specific banding patterns and allowed the unambiguous

individual identification of all the chromosome pairs making possible the preparation of accurate karyotypes and their respective ideograms (Figs. 1–4). Therefore, this technique has demonstrated to be a reliable technique and more prompt (compared with conventional banding techniques) for veneroid chromosome banding.

For the construction of the ideograms, we only described the presence of the bands and each band's relative position; the intensity of the bands was not considered. The intensity of the bands in the RE treatments seems to be related to the type of counterstain used (e.g., Giemsa or fluorochroms) (Gonsálvez et al. 1991). Several authors demonstrate that the loss of DNA after a RE digestion can increase the capacity of the stain to bind to a specific chromosome region (Gonsálvez et al. 1991, Nieddu et al. 1999). Therefore, it seemed reasonable not to consider the intensity of the bands in the construction of ideograms but only their presence and position.

The *in situ* restriction banding technique, applied here to both

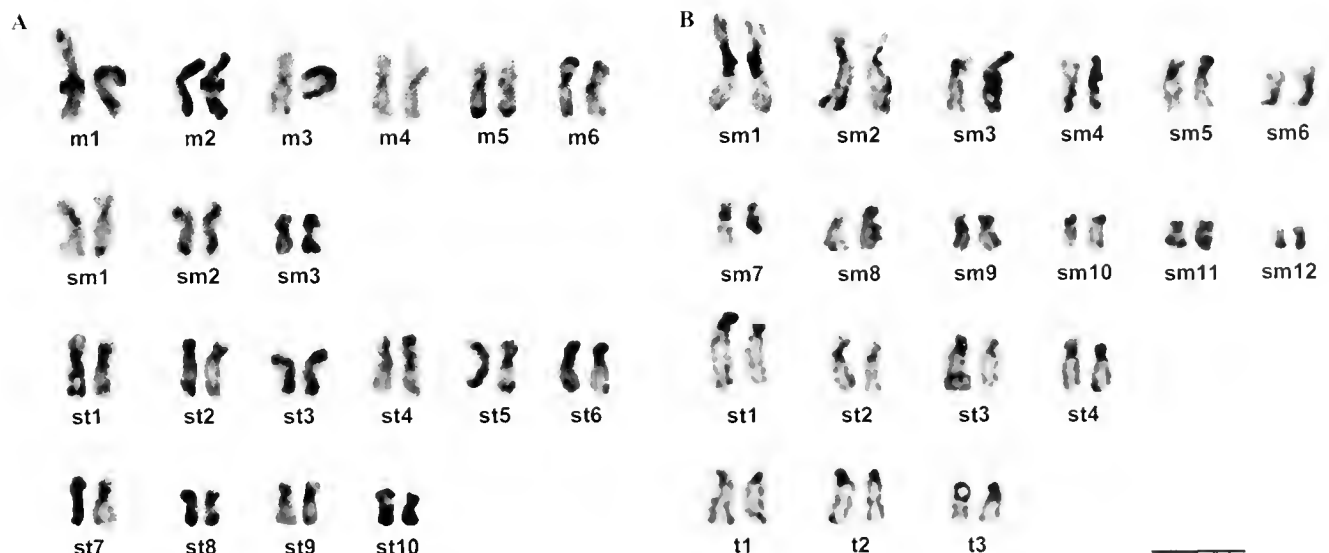


Figure 2. Examples of diploid-banded karyotypes. Figure 2a, diploid karyotype of *R. decussatus* banded with HaeIII, Figure 2b, diploid karyotype of *C. edule* banded with HaeIII. These examples are presented, to show the banding pattern consistency between homologous pairs. Scale bar = 5  $\mu$ m

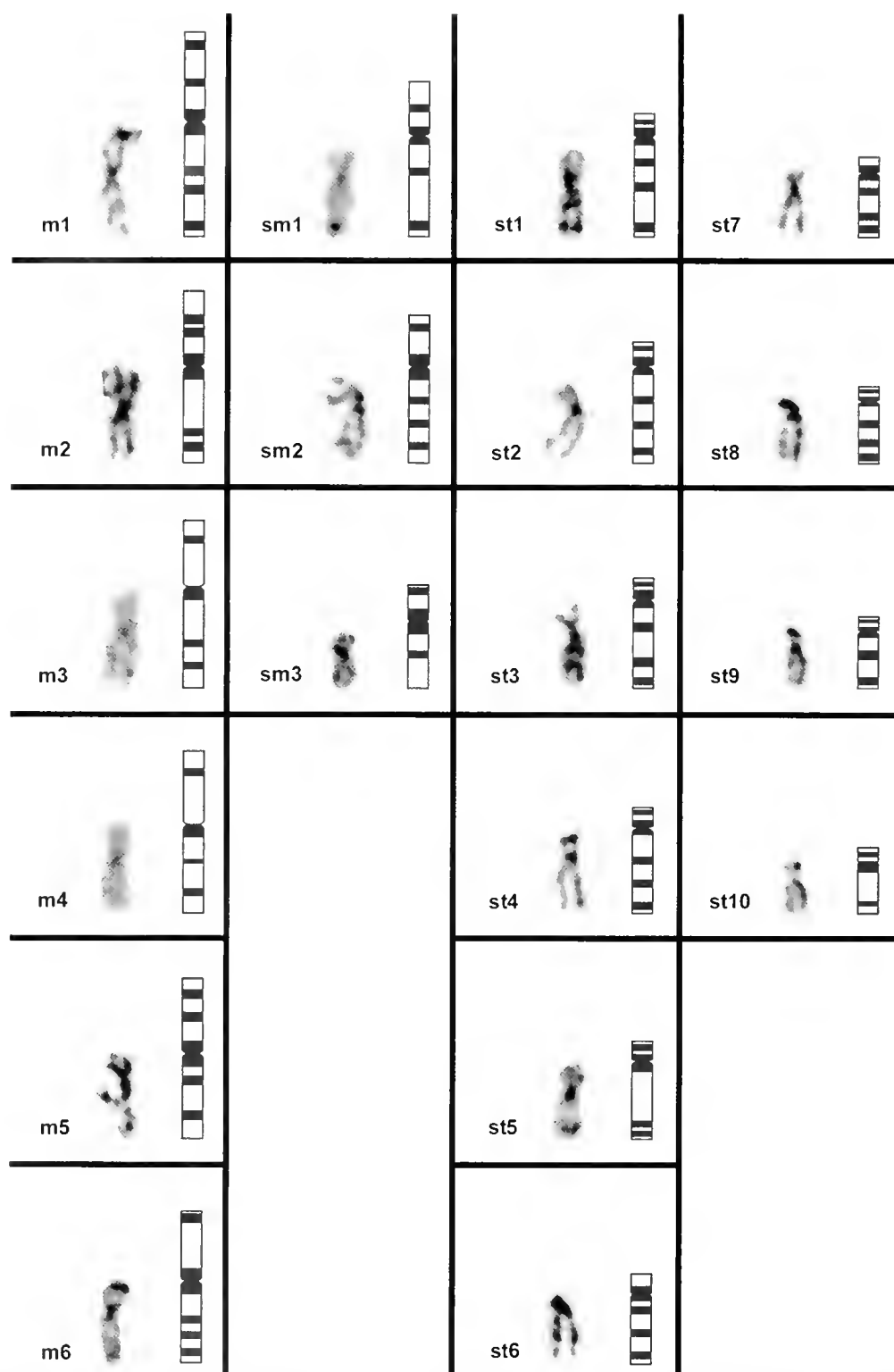


Figure 3. Haploid distribution of the chromosomal bands (left side of each column) and schematic representation of the *in situ* restriction banding patterns (right side of each column) obtained for the RE HaeIII in *R. decussatus*.

species, presents a major advantage, that can be used simultaneously with FISH techniques (Chaves et al. 2002), demanding only one round of observation and minimal extra preparation steps. Consequently, the *in situ* restriction banding technique will facili-

tate physical mapping in this group of bivalves, besides being compatible with more traditional banding techniques.

Furthermore because tissue culture protocols are not yet available in marine bivalves, the chromosomes are prepared directly

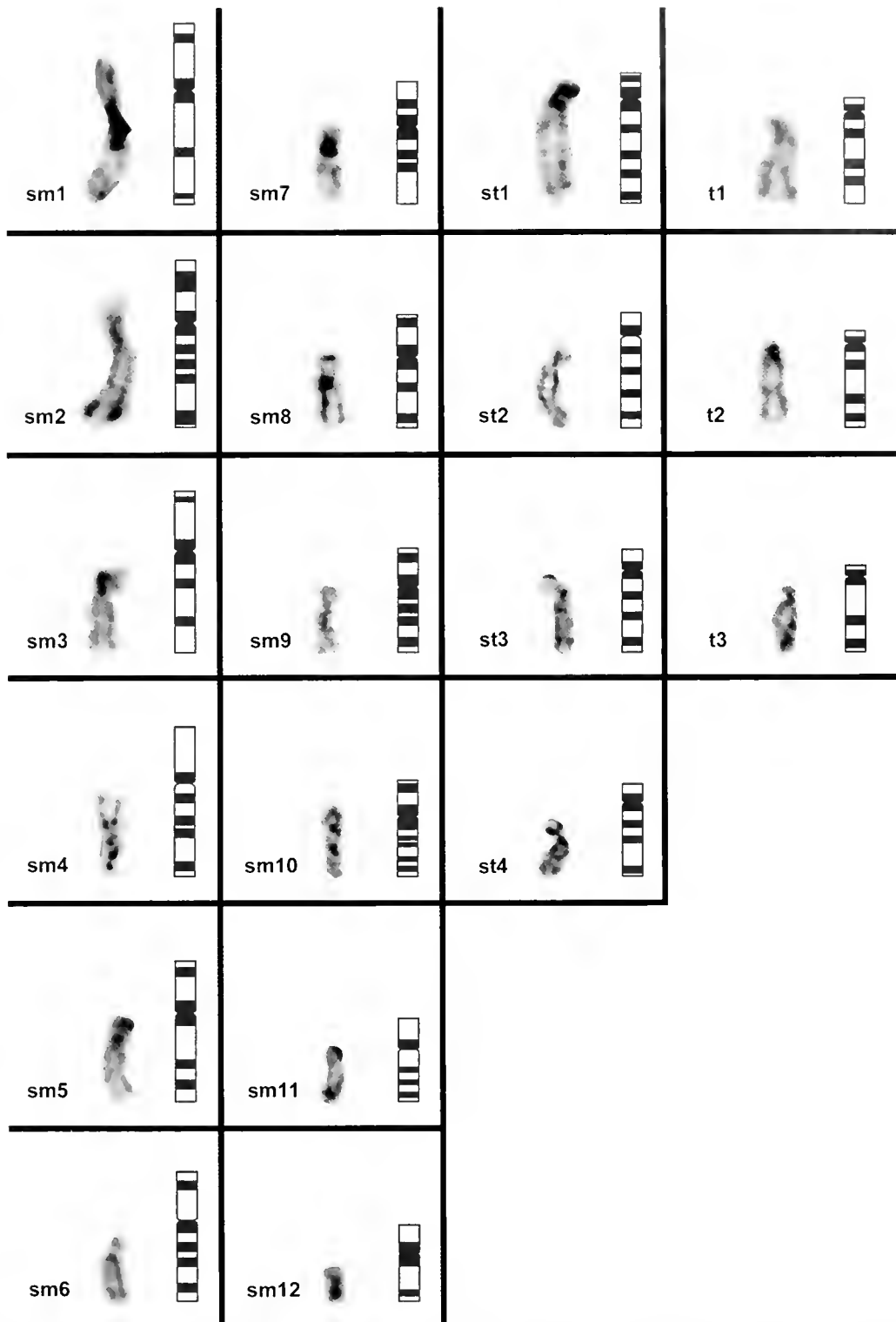


Figure 4. Haploid distribution of the chromosomal bands (left side of each column) and schematic representation of the *in situ* restriction banding patterns (right side of each column) obtained for the RE Hae III in *C. edule*.

from the animals and are of poor morphology. The *in situ* restriction banding technique better preserves the morphology of the chromosomes (compared with other conventional banding methods), representing an additional advantage for the identification of

veneroid chromosomes, when using further techniques such as FISH or C-banding (Chaves et al. 2002).

The use of the RE-banding technique can be very useful in several studies of economic or ecological importance within this

group of veneroid bivalves. For instance: (a) in evolution of chromosome and karyotypes; (b) can also provide a rapid method for the identification of the missing chromosomes in aneuploidy situations, for which a negative correlation with the growth rate was put in evidence in other bivalve species (Leitão et al. 2001) and (c) for the study of the impact of contaminants (anthropogenic compounds, and so forth) on the genetic patrimony of veneroids (through the identification of possible neoplasias, missing chromosomes, deletions, translocations and so forth). This last application is far more important because the clam *R. decussatus* has been recently proposed as a potential bio-indicator species in areas where mussels are not available (Bebianno et al. 2004). In fact clams are appropriate organisms for monitoring because they are sedentary filter feeders that exhibit a high level of diversity at a large number of loci (Moraga et al. 2002). Moreover, the tissue most currently

used for cytogenetic analysis is from the gills, which is one of the most "interesting" tissues from the ecotoxicological point of view (Bebianno et al. 2004).

This study shows that the applications of restriction enzyme chromosome banding in veneroids are diverse and can constitute a fundamental step in gene mapping in this commercially important group of bivalves and could offer a new approach to specific problems in veneroid taxonomy and genetics.

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## THE HYPOXIC TOLERANCE OF THE PROTOBRANCH BIVALVE *NUCULA SULCATA* BRONN

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**ABSTRACT** Examination of the hypoxic tolerance of *Nucula sulcata* determined that the mean survival time under hypoxia was 9.57 d with a maximum longevity of 30 d. Comparison between the three species, *N. sulcata*, *N. nitidosa*, and *N. nucleus* reveals that *N. sulcata* is the most tolerant of hypoxia followed by *N. nucleus* and *N. nitidosa*. It is suggested that the hypoxic tolerance of the species is a reflection of the normal habitat they occupy.

**KEY WORDS:** environmental tolerance, hypoxia, *Nucula sulcata*, protobranch, adaptation

### INTRODUCTION

The protobranch bivalves, *Nucula sulcata*, *N. nucleus*, and *N. nitidosa* are three of the five species of Nuculidae commonly found in soft sediments in British waters. Their distribution has been linked to particular sediment types, with *N. nitidosa* inhabiting muddy sand; *N. nucleus*, muddy gravels; and *N. sulcata* sandy mud/mud, hence in most locations only one of the species is found. This has led to the suggestion that the distribution of the species is determined, in part, by their physiology. For example, *N. nitidosa* and *N. nucleus* have been shown to have different metabolic rates, which can be related to their particular habitats (Holmes et al. 2002, see also Wilson & Davies 1984, Creutzberg 1986).

One important adaptation between the three species, that may affect their distribution, is their tolerance to hypoxia. With regard to *N. nitidosa* and *N. nucleus*, it has been determined that *N. nucleus* has a hypoxic tolerance ~3 times that of *N. nitidosa*, which may be reflected in its occupation of muddier habitats and hence an increase in its likelihood of experiencing hypoxic events (Holmes et al. 2002). The only observation pertaining to the hypoxic tolerance of *N. sulcata* is effectively anecdotal in nature (Taylor et al. 1995) with no replication and/or independence of experimental design, as is the case for most hypoxic tolerance studies (see Dries & Theede 1974 and Zwaan et al. 2001 for examples). The aim of this study, was to measure the hypoxic tolerance of *N. sulcata*, using an experimentally robust design and directly compare these results with those obtained for *N. nitidosa* and *N. nucleus* in earlier years, previously published elsewhere (Holmes et al. 2002), under the hypothesis that the hypoxic tolerance of the species should increase as the mud content of their habitat increases (i.e. as the likelihood of the frequency of hypoxic events increases).

### MATERIALS AND METHODS

Bivalves, *N. sulcata*, were collected in the mouth of Gullmarsfjord, off Gäsö island (58°14.19'E 11°26.36'N) in 2001, using an Agassiz trawl, and taken to Kristineberg Marine Research Station, Fiskerbackskil, Sweden where all experiments were carried out. Once at the laboratory, the specimens were left for 5 days in sediment to acclimatize, in running seawater (~10°C). Measurement of the hypoxic tolerance of *N. sulcata* was made as follows (the measurement of the hypoxic tolerance of *N. nitidosa* and *N. nucleus* was made in an identical fashion and is reported elsewhere [Holmes et al., 2002]). Ten liters of autoclaved filtered (0.2 µm) seawater (10°C) was vigorously bubbled with filtered (0.2 µm) N<sub>2</sub>, in a semi-enclosed vessel, for 6 h prior to the start of the

experiment (i.e., to ensure that the seawater contained as little oxygen as possible). One hundred and twenty, 9-mL (nominal) bottles were then filled with the seawater and a randomly selected animal placed into each. Ninety of the bottles were then capped, ensuring that the bottle remained under the surface of the water at all times (treatment animals), and the remaining 30 bottles were left uncapped (control animals). In addition, 35 bottles were filled with seawater and the bottles capped (water control). Five of these bottles were immediately analyzed for their oxygen content (Holmes et al. 2002). All of the bottles were placed together under a 12 h day/night cycle and maintained at 10°C for the duration of the experiment.

Every day, the treatment and control animals were inspected, individually, to ascertain whether they were alive. If an animal was found to be dead, it was removed from the experiment and its death recorded along with its shell height, width, length, whole dry weight, flesh dry weight, and the oxygen concentration of the water. Every 5 days, from the start of the experiment, 5 randomly selected water controls were removed and the oxygen concentration determined. The end of the experiment was determined by the death of all of the animals. It should be noted that, the measurements of hypoxic tolerance made here will inherently include other confounding factors (e.g., starvation) that will result in an under, rather than over estimation.

### RESULTS AND DISCUSSION

The mean oxygen concentration of the seawater at the start of the experiment ± standard error (SE) was  $1.56 \pm 0.13$  mL O<sub>2</sub> l<sup>-1</sup> and hence the seawater can be regarded as hypoxic at the start of the experiment, see Figure 1 (Diaz & Rosenberg 1995). Comparison of the control water oxygen concentration during the experiment, using ANOVA, revealed no differences. Linear regression of the lifetime of *N. sulcata* versus the mean oxygen concentration of the water samples, at the time of death, produced a statistically significant regression. The O<sub>2</sub> concentration of the water for the treatment animals dropped over time as they respired, leading to their eventual death (Fig. 1). Analysis of the number of animals dying per day, against the mean O<sub>2</sub> concentration for the animals that died that day, using Spearman's rho, to determine if there was a threshold O<sub>2</sub> concentration lower than that at the start of the experiment that caused death, produced no statistically significant correlation: the initial O<sub>2</sub> concentration of the water was sufficient without any further reduction in its oxygen concentration to cause death by hypoxia. Correlation, using Spearman's rho, of the shell length, height, width, whole body dry weight, and dry flesh weight data to the survival data, failed to produce any statistically significant correlations (i.e. death caused by hypoxia does not seem to be related to body size).

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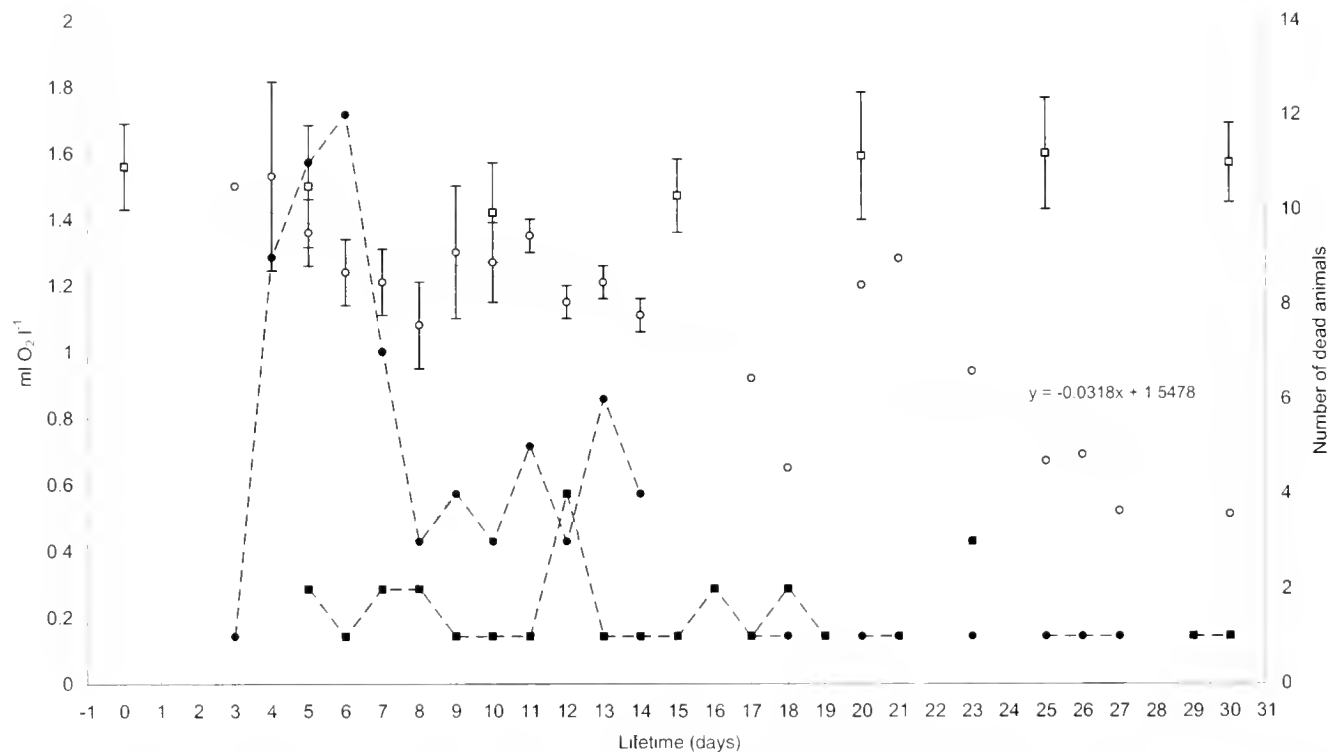


Figure 1. The mean and standard error of the oxygen concentration of the control and treatment samples compared with the number of *N. sulcata* that have died on each day.  $\square$ , Mean oxygen concentration of the control samples;  $\circ$ , mean oxygen concentration of the treatment samples;  $\bullet$ , number of treatment animals that have died;  $\blacksquare$ , number of control animals that have died; calculated regression line (mean oxygen concentration treatment samples).

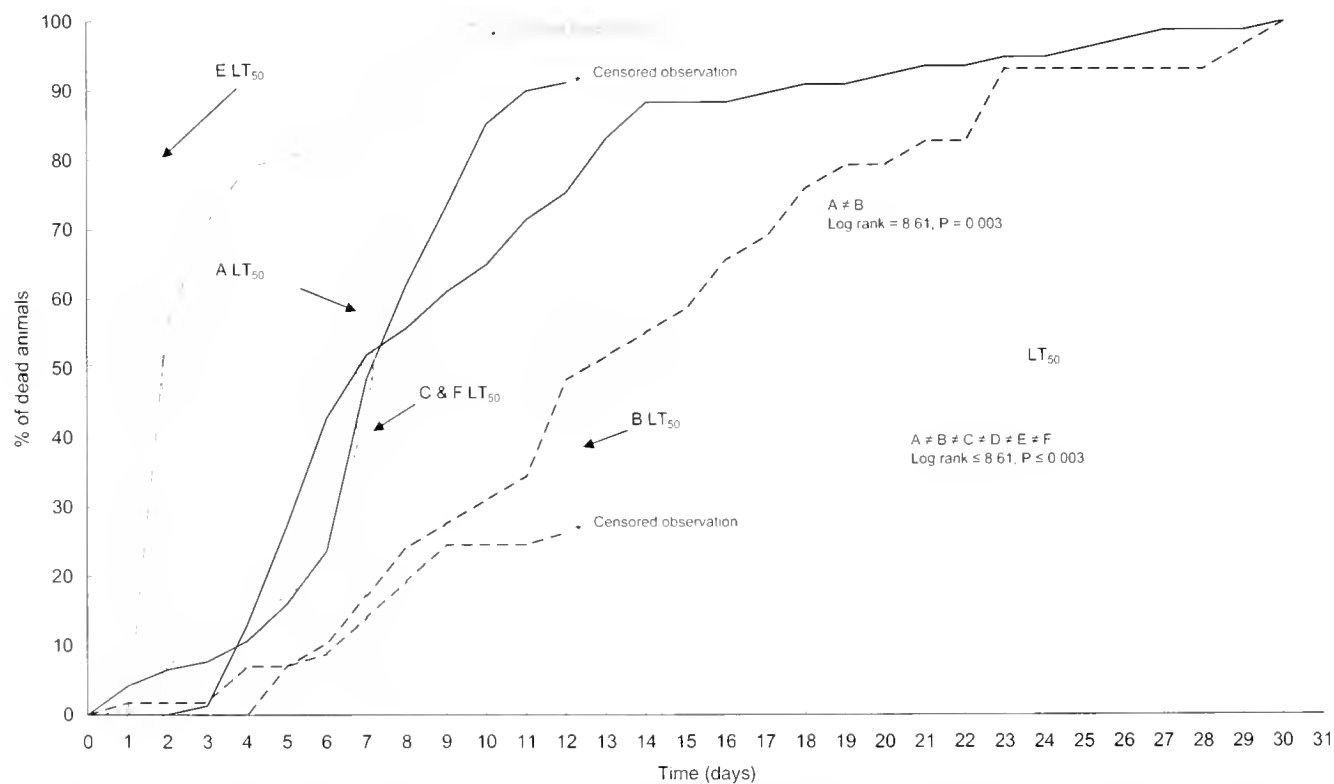


Figure 2. Comparison between the death rate (cumulative percentage) of *N. sulcata*, *N. nucleus*, and *N. nitidosa* treatments and controls. — (A) Treatment *N. sulcata*; --- (B) Control *N. sulcata*; — (C) Treatment *N. nucleus*; --- (D) Control *N. nucleus*; ···· (E) Treatment *N. nitidosa*; ···· (F) Control *N. nitidosa*.

Analysis of the survival data, using the Kaplan-Meier procedure with log rank comparisons (Klein & Moeschberger 1998), determined that survival rate of the *N. sulcata* treatment animals was different from that of the *N. sulcata* control animals ( $P = 0.003$ , Fig. 2). The mean  $\pm$  SE survival time for the treatment animals was  $9.57 \pm 0.68$  d (median = 7 d,  $LT_{50} = 7$  d) and  $14.45 \pm 1.27$  d (median = 13 d,  $LT_{50} = 13$  d) for the control animals (Fig. 2). For the treatment and control animal the maximum longevity was 30 d. Note, 13 treatment animals and one control animal bottles were broken during the experimental period and hence were excluded from the analysis. Comparison between the previously published survival data for *N. nitidosa* and *N. nucleus* with the *N. sulcata* data, again using the Kaplan-Meier procedure with log rank comparisons, revealed that survival rates for all species were different from each other ( $P \leq 0.003$ ) (Fig. 2). That is, *N. nitidosa* (mean  $\pm$  SE survival time =  $3.53 \pm 0.28$  d) was most susceptible to death by hypoxia followed by *N. nucleus* (mean  $\pm$  SE survival time =  $7.72 \pm 0.21$  d) and then by *N. sulcata*. In effect, *N. sulcata* has a hypoxic tolerance  $\sim 1.25$  times greater than that of *N. nucleus* and 5 times that of *N. nitidosa*. In terms, of total longevity (i.e., the time taken for all animals to die), *N. sulcata* has a maximum longevity of approximately twice that of *N. nucleus* (longevity  $\sim 15$  d) and approximately three times that of *N. nitidosa*. The maximum survival time recorded for *N. sulcata* (30 d) is less than that recorded, but of the same order, for *Corbula gibba* (34 + d), which occupies a similar habitat (Holmes & Miller 2006).

With regard to the existing observation within the literature as to the hypoxic tolerance of *N. sulcata* ( $LT_{50} = 14$  d and longevity = 21 d) (Taylor et al. 1995) the values reported here are different. In part, arising from the different methodologies used but also from the more robust experimental design adopted. Irrespective of the cause of the difference, the results presented here for *N. sulcata* are directly comparable with those recorded for *N. nitidosa* and *N. nucleus*, revealing as hypothesized that the hypoxic tolerance of the three species can be linked to the habitat they normally occupy. In terms of comparison with other nuculides, *N. tenuis* has been recorded as having a maximal longevity of 17 d (Moore 1931), and there are no observations for *N. hanleyi*. If the hypothesis substantiated were correct, then it would be interesting to measure the hypoxic tolerance of *N. hanleyi*, which should be approximately equal to or lower than that of *N. nitidosa* and *N. hanleyi* occupying sandy sediments with a high oxygen concentration. Similarly, the hypothesis could be expanded to a range of bivalve species, for example *Abra alba*, which inhabits sandy mud and has a much lower hypoxic tolerance than *A. elliptica* occupying muddy/gravelly sands (Dries & Theede 1974). The importance in individual, nonobservable, adaptations between closely related species in determining their distribution patterns has been often overlooked in sublittoral benthic ecology. However, any experiments performed to attempt to discern adaptational differences need to be done in an experimentally robust manner, with independent observations, such that correct cross comparisons between species can be attained.

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## REPRODUCTIVE CYCLE OF THE RAZOR CLAM *SOLEN MARGINATUS* (PULTENEY 1799) IN SPAIN: A COMPARATIVE STUDY IN THREE DIFFERENT LOCATIONS

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**ABSTRACT** The reproductive cycle of the razor clam *Solen marginatus* (Pulteney 1799) was studied in three different locations of Spain. Sampling was performed in the natural beds of: Eo Estuary (NW Spain) in 1994 to 1995, Santander Bay (N Spain) in 1998 to 1999 and Terrón Estuary (SW Spain) in 1999. In Eo, Santander and Terrón, respectively: phase 0 (resting stage) occurred in July to August, September to October and August to September, phase I (proliferation of gonias) happened in: August to October, October to December and September to November, phase II (gametogenesis) was developed in November to April, January to May and December to April and phase III (reproduction) took place in: May to June, June to August and May to July. Two new approaches oriented to the fast monitoring of the gametogenic cycle of the species based on sequential mean drained soft parts weights obtained by simulation and on the macroscopic observation of the gonad are presented.

**KEY WORDS:** bivalves, *Solenidae*, *Solen*, reproduction

### INTRODUCTION

The superfamily Solenacea is an infaunal soft bottom dwelling bivalve group consisting of the two marine families Solenidae and Pharidae (= Cultellidae, Cosel 1993).

Razor clams are bivalves whose commercial demand has notably increased in Spain in recent years (Remacha-Triviño 1996). As a consequence of this, the natural beds of these species can be subjected to overexploitation.

*Solen marginatus* (Pulteney, 1799) is a representative intertidal bivalve of the Spanish coasts. Accordingly, this species is distributed along the west Mediterranean Sea and Atlantic Ocean from Britain to Mauritania (Cosel 1993), comprising all of Spain's coasts. This species can be considered the third most important commercial razor clam of Spain after *Ensis arcuatus* and *E. siliqua*.

Previous references dealing with the reproductive cycle of *S. marginatus* are: Rodríguez-MoscOSO et al. (1996), Gaspar (1996), Remacha-Triviño (1996), Tirado et al. (2002), Remacha-Triviño (2002), Martínez (2002) and López et al. (2005). However, there are not precedent studies dealing with the reproductive cycle of this species either in Santander Bay or in Terrón Estuary, as well as a comparative survey among these three locations.

Two of the most important strategies oriented to improve the management of the marine medium are a more rational exploitation of the natural resources and the application of aquaculture as an instrument aimed to increase the production of commercial species. Alternatively, artificial culture constitutes a way to compensate the lack of other species, which, because of overharvesting continue to diminish. A thorough comprehension of the reproductive cycle provides: (1) the establishment of close seasons in keeping with the spawning periods; (2) optimization of the breeding conditions oriented towards improving the quality of commercial exploitation and, in a more general framework, with the purpose of expanding sustained development and (3) the genetic selection of varieties of a higher reproductive efficiency and more resistant to pathologies or stress conditions related to reproduction.

The purpose of this study is to draw a comparison among the reproductive cycle of *S. marginatus* in three separate locations of

Spain and to present two new approaches oriented to the fast monitoring of the gametogenic cycle of this species, based on the macroscopic observation of the gonads and on sequential mean drained soft parts weights obtained by simulation. In this way, the understanding of the reproductive biology of *S. marginatus* is improved, making this information relevant for promoting a future extensive culture for this species, oriented to satisfy its commercial demand and to guarantee the conservation of its natural beds.

### MATERIALS AND METHODS

Samples of 50–65 razor clams were collected intertidally in 3 different locations including the Eo Estuary (Asturias, NW Spain), Santander Bay (Cantabria, N Spain) and Terrón Estuary (Huelva, SW Spain) (Fig. 1). Specimens from Eo Estuary were collected from June 1994 to May 1995. Specimens from Santander Bay were collected from October 1998 to September 1999. Specimens from Terrón Estuary were collected from January 1999 to December 1999.

Live razor clams were transported to the Invertebrates Laboratory of the Department of Biology of Organisms and Systems (University of Oviedo, Asturias, Spain). Caliper shell lengths and drained weights were measured to the closest 0.01 mm and g, respectively. Next, animals were fixed in 10% formalin in seawater and the following procedures were applied.

#### Histological Techniques

Twelve animals (six males and six females) per month were used for histology. A sample of approximately 10 × 5 mm visceral mass was dissected from the internal ventral portion of each foot (Remacha-Triviño 1996), starting from a fixed point (i.e., left square) chosen at random, dehydrated in graded ethanols and embedded in paraffin according to Durfort (1993). Seven micrometers serial sections were obtained and stained routinely with Hematoxylin-Eosin. Stages of the reproductive cycle were classified under the maturity scale of Chipperfield (1953) and Lubet (1959) widely used by different authors (e.g., see Villalba 1995), which comprises the following phases: 0 (resting stage), I (proliferation of gonias), II (gametogenesis) and III (reproduction) of subphases: III A (ripeness), III B (early spawning), III C (restoration) and III D (late spawning).

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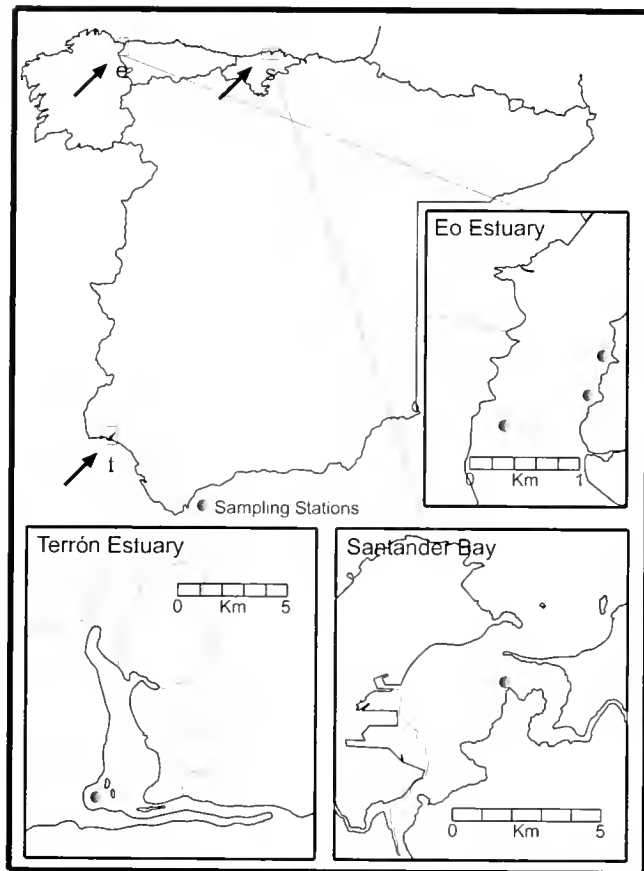


Figure 1. Map of western Spain (excluding Portugal). Arrows show the relative locations of: Eo Estuary (NW Spain), Santander Bay (N Spain) and Terrón Estuary (SW Spain). Abbreviations: e, Eo Estuary; s, Santander Bay; t, Terrón Estuary.

#### Macroscopic Observation of Gonads

Feet were dissected ventrally along the line of intersection with the sagittal plane. Gonads were classified in relation to their macroscopic appearance under the maturity scale used for histology.

#### Sequential Mean Total Drained Weights

Sequential mean total drained weights were estimated in each month from the arithmetic progression of shell lengths restricted to sizes between 65–130 mm,  $\{a_L\} = 5 \cdot (12 + L) \nabla_{a_L} [65,130]$ , whose elements were obtained by simulation from their corresponding monthly regressions  $\log W | \log L$ .

### RESULTS

#### Extension of the Gonad

The extension of the gonad of *Solen marginatus* is restricted to the internal portion of the foot where it is enclosed permanently, even in the periods of maximal gonadal development. Follicles line the internal surface of the pedal sinus, adjacent muscular layers and parts of viscera enclosed therein and facing its lumen. The interfollicular connective tissue, common in most bivalve species, is absent.

#### Histology

##### Phase 0 (Resting Stage)

Sex Indistinguishable. Spread of follicular tissue limited to minor areas located among the peripheral muscular bundles of the

pedal sinus (Fig. 2A). Gonads were not observed in some of the specimens. Hemocytes surrounding the unabsorbed necrotic masses derived from the precedent subphase III D (see below) were frequent. Gametogenic elements reduced to reservoirs of stem cells (Fig. 2B to C) of characteristics: (1) ellipsoidal, spheroidal or polygonal shapes; (2) basophilic to lightly acidophilic staining affinity; (3) bigger size than gonias; (4) high nucleocytoplasmic quotient and (5) boundary cytoplasm-nucleus diffuse.

##### Phase I (Proliferation of Gonias)

Intense mitotic activity and spread of gonias, which proliferated by forming disperse poolings of cells located around the internal muscle layers and visceral mass of the foot (Fig. 2D to G). Clusters of gonias were enclosed within the follicular cells to constitute the primary follicles. Oogonias and spermatogonias showed common morphological features. Both cells were roughly ellipsoidal, displaying neutrophil cytoplasm, slightly acidophilic nuclei, clear boundaries nucleus-cytoplasm and similar sizes, ranging between 3 and 13  $\mu\text{m}$  in diameter (Fig. 2E, G, L).

##### Phase II (Gametogenesis)

In females, the first previtellogenic oocytes were observed at the beginning of this phase. When compared with oogonias, the former exhibited cytoplasm and nuclei with a similar degree of neutrophilia and a diffuse boundary cytoplasm-nucleus with no visible nucleolus. Some of these first oocytes remained free in the follicular lumen, with no apparent point of attachment to the follicular wall (Fig. 2J). In posterior stages of oogenesis, nuclear membrane and nucleolus became distinguishable and oocytes were found either attached to the follicle wall or free in the follicular lumen, but always connected to aerial follicular cells in the latter case (Fig. 2H, I). In males, spermatocytes were the predominant cellular type in early spermatogenesis (Fig. 2K). These were roughly spheroidal cells of approximately 5  $\mu\text{m}$  in diameter, deep basophilic nuclei and a high nucleocytoplasmic quotient. In posterior phases of early gametogenesis, the gonad gradually invaded the internal muscular and fibrous connective surfaces of the pedal sinus and free portions of the viscera—digestive gland and gut—enclosed within the foot, with the exception of some concrete areas lined exclusively with integumentary musculature. In winter, the progress of the gametogenesis was generally slow or in recession. Late gametogenesis was mainly developed through spring. In females, oocytes nuclei tended to emigrate towards a more apical position within the cytoplasm, whereas the longitudinal axis of the cells was used to reallocate radially towards the follicular lumen. Oocytes were generally attached to the follicular wall by means of stalks that were not as evident as in other bivalve species. Masses of basophilic clusters of atresic oocytes were frequent. In males, proliferation of spermatocytes was increased notably, a process simultaneous with the first manifest masses of spermatozoa. Male gonads were composed mainly of spermatocytes, spermatids and spermatozoa (Fig. 2L). Although spermatocytes continued to be the most frequent spermatogenic stage, densities of the other cellular types were augmented significantly. The first minor releases of gametes took place at the end of this phase.

##### Phase III (Reproduction)

**Subphase III A (Ripeness):** Gonads were deeply packed and full of gametes near emission. Follicles were extremely expanded. In most cases, the feet became so dilated that they were hardly



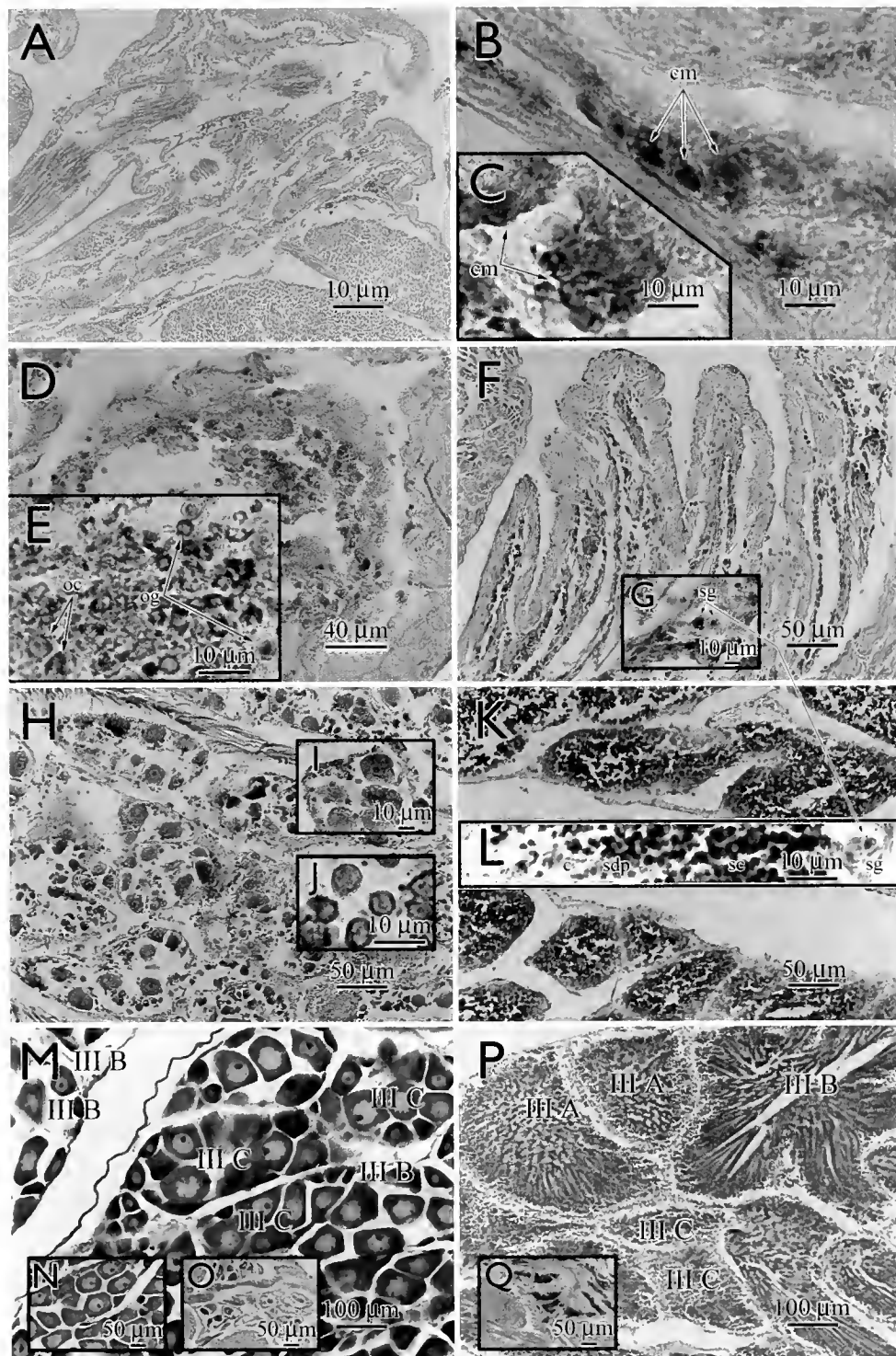


Figure 2. Photomicrographs of gonadal stages of *S. marginatus*. A: Phase 0. Sex undetermined. B to C: Phase 0. Clusters of stem cells. Sex undetermined. D: Ovary in phase I. E: Detail of D showing oögonias and the initial stages of the oöcytes (note the similarity of these first oöcytes with the first oöcytes presented in J). F: Testis in phase I. G: Detail of F showing spermatogonias (note the similarity of these spermatogonias with the spermatogonias presented in L). H: Ovary in phase II. I: Detail of H showing the junctions of the oöcytes to the follicular cells. J: Detail of H showing the first stages of the oöcytes. K to L: Testis in phase II. M: Ovary in subphases III B and III C, simultaneously. N: Ovary in subphase III A. O: Ovary in subphase III D. P: Testis in subphases III A, III B and III C, simultaneously. Q: Testis in subphase III D. Abbreviations: e, tails of spermatozoa; sdp, spermatids and heads of spermatozoa; cm, stem cells; oc, oöcytes; og, oögonias; sc, spermatocytes; sg, spermatogonias.

retractable. Atresic oöcytes were less frequent than in phase II, as indicative of a probable mechanism of resorption. Mature oöcytes were detached from the follicle wall, adopting a more spheroid

geometry turned to poliedric at highly packed areas (Fig. 2N). Males showed a significant predominance of spermatozoa mainly positioned at the center of the follicular lumen (Fig. 2P).

**Subphase III B (Spawning):** Phase of spawning was restricted to unspecific areas of the gonad, whose follicles were found to be partially evacuated because of the releases of gametes (Fig. 2M, P).

**Subphase III C (Restoration):** Intervals among consecutive spawnings were characterized by a restorative activity yielded to a highly variable pattern, depending on sex, number of precedent spawnings and duration of these previous periods of spawning. In females, restoration gave rise to follicles whose oocytes showed a major proportion of immature oocytes. Spreading of oogonias was also observed. In males, the follicles found in coexistence with follicles III A and III B, with no spaces in the lumen and without a predominance of spermatozoa, were classified under phase III C, although the pattern of restoration was not clearly observed in males (Fig. 2M, P).

**Subphase III D (Last Emissions):** Evacuation of all the potentially releasable gametes from the gonad generally happened after a succession of various minor spawnings plus a major one. Nevertheless, gonads examined after the major spawning showed follicles that were not completely evacuated, implying some minor releases after the major spawning to complete this process. In the evacuated follicles, elements of gametogenesis were reduced to small pools of gonias, stem cells and necrotic masses of residual oocytes. A strong parallel resorptive activity took place. Follicles were progressively drawn back down the fibromuscular tissues and reabsorbed later (Fig. 2O, Q). Groups of hemocytes were particularly numerous in this phase by forming aggregates of phagocytosis within the degenerating tissues.

Sequential ratios of phases of the reproductive cycles investigated are presented in Figures 3, 4, 5.

#### Macroscopic Level

Phases 0 and I. Gonad not observed macroscopically or reduced to a thin layer of hyaline tissue. Whitish areas corresponding to putative primordia of follicular tissue were detected occasionally over the internal surface of the pedal sinus (Fig. 6A to B). Phase II. Gonad clearly observed macroscopically, extending from discontinuous portions of the pedal sinus to almost all its inner surface area. Internal surface of the gonad mainly distributed in form of small transverse folds of tubular appearance. These folds were whitish in males and brownish in females (Fig. 6C to D). Subphases III A, III B and III C. Pedal sinus almost obliterated by gonad. Internal surface of gonad composed by bigger folds resem-

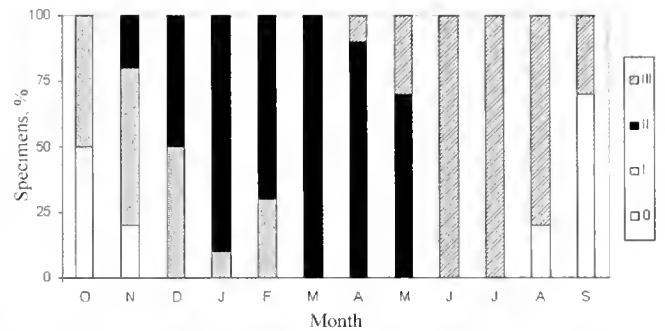


Figure 4. Sequential ratios of phases of the reproductive cycle of Santander Bay observed in histological sections. Abbreviations: 0, phase 0; I, phase I; II, phase II and III, phase III.

bling small sacs of fragmented surface in males and wavy surface in females (Fig. 6E to F). Subphase III D. (1) Early stage: Internal surface of gonad arranged in folds similar to the previous ones but less dimensioned. (2) Late stage: Gonadal arrangement analogous to phase II except for the inside of the tubular folds, which are covered in stains, as a probable consequence of the mechanism of resorption (Fig. 6G, H).

#### Mean Total Drained Weight

The sequential mean total drained weights of the different reproductive cycles investigated are shown in Figure 7.

## DISCUSSION

#### Histology

The reproductive cycle of *Solen marginatus* fits the general pattern observed in other bivalves of temperate waters (i.e., annual cycle, seasonal phases, all the different phases cannot be found simultaneously) being analogous to: *Crassostrea virginica*, *Cerastoderma edule*, *C. glaucum*, *Mytilus edulis*, *M. galloprovincialis*, *Lithophaga lithophaga*, *Paphies australis* and *Venus striatula* (Loosanoff 1942, Lubet 1959, Ansell 1961, Boyden 1971, Galinou-Mitsoudi & Sinis 1994, Giguère et al. 1994, Villalba 1995), among other species.

The evolution of the reproductive cycle of *S. marginatus* is, likewise, globally coincident with other references on this species (Rodríguez-Moscóso et al. 1996, Gaspar 1996, Gaspar & Monteiro 1998, Tirado et al. 2002, Martínez 2002, López et al. 2005) and other Solenidae (Martínez et al. 1997, Casavola et al. 1985, Darriba et al. 2004); with the exception of the well-known expectable

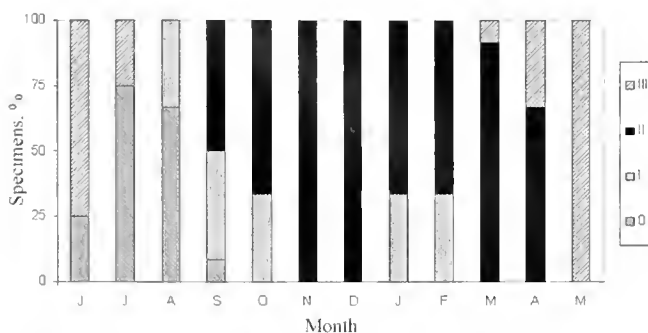


Figure 3. Sequential ratios of phases of the reproductive cycle of Eo Estuary observed in histological sections. Abbreviations: 0, phase 0; I, phase I; II, phase II; III, phase III.

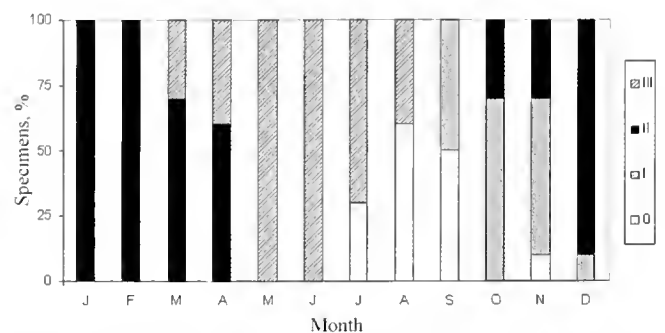


Figure 5. Sequential ratios of phases of the reproductive cycle of Ter-rón Estuary observed in histological sections. Abbreviations: 0, phase 0; I, phase I; II, phase II and III, phase III.

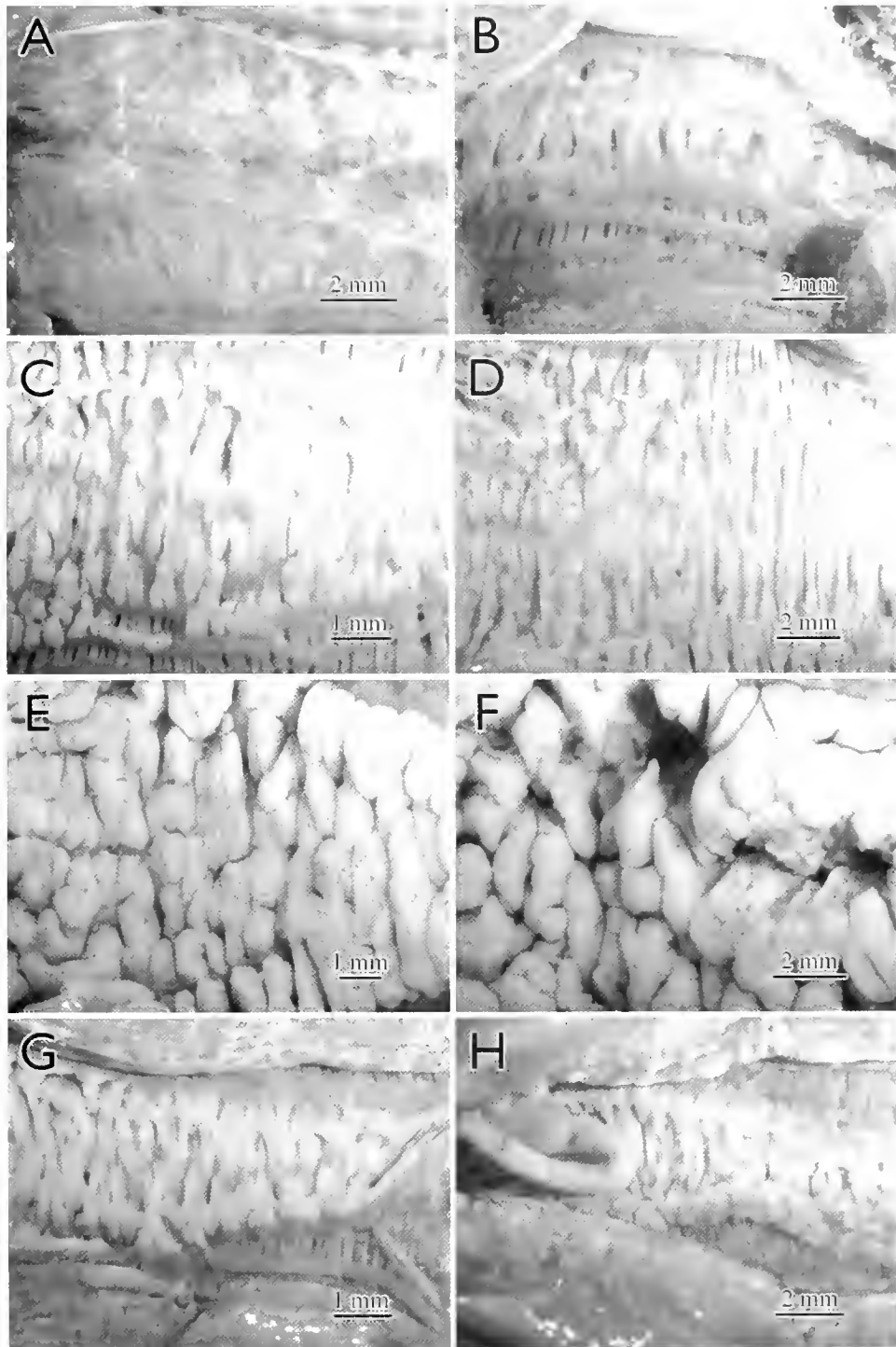


Figure 6. Macroscopic gonadal stages of *S. marginatus*. A to B: Phase 0 and I, indistinctly. Sex undetermined. C: Female gonad in phase II. D: Male gonad in phase II. E: Female gonad in phases III A to C. F: Male gonad in phases III A to C. G: Female gonad in phase III D. H: Male gonad in phase III D.

differences in duration and occurrence of the different phases of the cycle due, on the one hand, to feeding, temperature, salinity, photoperiod and, in general, other potentially influent environmental conditions; whereas on the other hand, to probable genetic variations, specially from the Southern population of Terrón Estuary with respect to the northern ones of Eo Estuary and Santander Bay. The aforementioned deviations are also expected

to explain the differences among the three reproductive cycles investigated here.

Spermatogonias and oögonias were analogous to other bivalves (Tranter 1958, Lubet 1959, Darriba et al. 2004). However, some differences with respect to *Ensis arcuatus* were found. Although protogonias have been described in other bivalve species as cells of similar size to oögonias (De Gaulejac et al. 1995), Darriba et al.

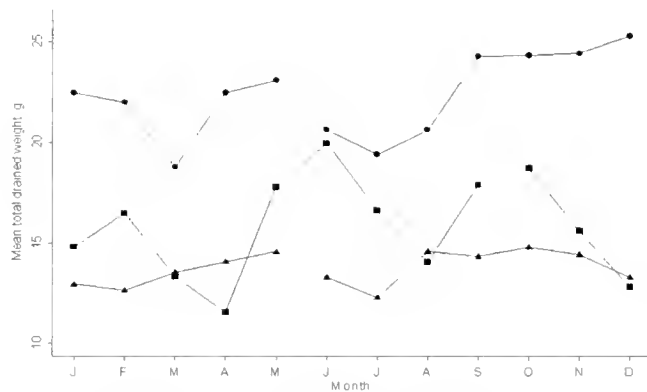


Figure 7. Mean total drained weights from: Eo estuary (June to December 1994 and January to May 1995) (triangles); Santander Bay (October to December 1998 and January to September 1999) (squares); Terrón Estuary (January to December 1999) (points).

(2004) described the protogonias of *E. arcuatus* as acidophilic cells bigger than oögonias and reported larger and less basophile oögonias than spermatogonias. Nevertheless in *S. marginatus*, oögonias and spermatogonias showed similar dimensions and degrees of acidophilia.

Distinction between spermatogonias and oögonias in phase I was carried out by the observation of specimens in the transition between the former phase and phase II, in which the subsequent gametogenic stages (i.e., first oocytes and spermatocytes) were clearly observed.

Subphases III A (ripeness), III B (spawning), and III C (restoration) were found simultaneously in only a few animals (Fig. 2, P). In the rest of specimens, two of these three subphases III A to C were always coexisting (Fig. 2M). In consequence, and unlike subphase III D and rest of phases of the reproductive cycle, we did not find razor clams in which the whole follicles were in the same subphase III A to C. Although the decision of classifying the specimens in phase III under one of these three subphases can be based on the state of most of the follicles, subphases III A, III B and III C are not always easy to distinguish in both sexes, as in females: (1) is unusual to find oocytes III A in absence of immature oocytes attached to the follicle wall, and therefore, involved in a simultaneous mechanism of restoration; (2) follicles III A showed frequent intrafollicular spaces, as indicative of a previous spawning. In this way, the loss of the polygonal shape generally observed in the mature oocytes of bivalve mollusks (e.g., Lubet 1959) can be explained as the consequence of a lower intrafollicular compression; (3) follicles III C are also used to show mature oocytes in the lumen. Alternatively, in males: (1) because the production of sperm could be predominant or balanced with production of spermatocytes during subphase III C, it is not clear that in *E. arcuatus* the process of restoration implies, as affirmed by Darriba et al. (2004), a necessary predominance of spermatocytes against spermatozoa in the follicle; (2) in phase II, we observed follicles with a minor proportion of sperm. If we define the mature male follicles as those with predominance of spermatozoa (e.g., Loosanoff, 1942) then the process of maturation of these follicles could have been reached after the emission of at least a portion of the sperm generated during phase II, giving rise to the frequently observed male follicles with broad luminal spaces, which are potentially classifiable under subphase III B. In synthesis, we define subphases III A to C as substages of the reproductive cycle of

frequent coexistence. Therefore, they should be classified more properly at the follicular level than at the individual level, as displayed in Figure 2M, P.

In this study, the gonad of *S. marginatus* was found to be located exclusively inside the foot during all phases of the reproductive cycle. Therefore, our results contradict the findings of Rodríguez-Moscó et al. (1996), authors who affirmed that in *S. marginatus*, the gonad entered the foot during the maturation period.

The function of hemocytes as reserve cells has been investigated by Houtteville (1974) and Medhiboub & Lubet (1988) for the species *Mytilus edulis* and *Ruditapes philippinarum*, who found a set of different transitional stages to reach the final reservoir stage termed vesicular cell. Rodríguez-Moscó et al. (1996) reported a prevalence of glycogen in the muscular tissues and digestive gland of *S. marginatus* and showed that this polysaccharide was poorly represented in the gonad. On the contrary, contents in lipids ranged between 16% and 30%. Remacha-Triviño (2002) found a parallel disorganization and subsequent reorganization of nephridia and gonads in *S. marginatus*, which was hypothesized as an indicative of a probable renal cycle simultaneous to the reproductive cycle. Particularly, the number of hemocytes of the distal limb of the nephridia was demonstrated by Stereology to be significantly increased in phase III. Most of these blood cells were observed to form phagocytic aggregates around the degenerating excretory cells. All these findings were interpreted as a probable mechanism of hemocytic transport of reservoir substances stored in the kidney to the gonad, where hemocytes would tend to remain accumulated as reservoir cells. Darriba et al. (2004) reported a probable feeding activity of the hemocytes of *Ensis arcuatus* by supplying nutrients and carrying out the function of reserve cells in the gonad.

#### Macroscopic Observation of Gonads

The observation of gonads performed at the macroscopic level permits fast approximations of the reproductive condition and to determine the sex of the specimens in the late stages of the reproductive cycle. Although the macroscopic description of gonads is a general complement to the histological approach in studies dealing with reproduction of bivalves, only a few maturity scales have been pursued in establishing a reliable parallelism at both levels of magnification through all the stages of the reproductive cycle in which this relationship was possible. In this context, the absence of structural perfollicular tissues in *S. marginatus* facilitated the design of a more precise macroscopic maturity scale for the species.

#### Mean Total Drained Weights

Biometric methods oriented to monitor the reproductive cycle can be classified in: (1) approaches based in one variable (i.e., soft parts weight, shell weight, total weight, soft parts volume, etc.); (2) condition indexes or functions of at least two variables; (3) standardizations derived from the extrapolation of data of (1) and (2) to a concrete value; (4) alternative simple statistical treatments (i.e., descriptive approaches, regressions, etc.); (5) complex statistical or biological treatments (i.e., temporal series or biological models).

Standardizations show the disadvantage of depending on the final value chosen for the extrapolation of data. Thus, results can only be compared in relative terms, unless the same standard value is used for all of the different treatments. In addition, standardiza-

tions would be recommended to be based in unbiased estimators, where the sample mean is likely to be the best and simplest election. Also, approaches (1) to (3) present the inconvenience of being range dependant. Therefore, a rational biometrical comparison should be expected to be derived from sequential range-balanced initial data. Although the previous selection of a fixed range for all the samples compared through time is, in practice, statistically questionable for the cases (1) to (3), as it means to lose the information of the discarded data, a common range can be achieved by statistical simulation (e.g., regression), or it can be chosen in terms of a rational criteria of election.

It is well known that one of the aims of condition indexes is to average the differences caused by size or range by dividing the characteristic of interest (e.g., dry soft parts weight) by a fixed variable, which is expected to remain invariant through time (e.g., dry shell weight). However, no effort is generally made in evaluating these variables separately. If it were so, it could be seen that the trends of some condition indexes widely used [e.g., Higgings' (1938), and Walne's (1970)] and the characteristic of interest can show an inverse relationship with respect to size. This fact can be easily verified by comparing the linear regressions between each one of the variables used in these condition indexes against a common characteristic implying dimension, alternative to the fixed one utilized in the condition index (e.g., for Walne's CI, dry soft parts weight versus shell cavity volume and dry shell weight versus shell cavity volume, since the inverse relationship for Higgings' CI). For instance, if we suppose that equations of the previous regressions were:  $Y = X + 1$  and  $Z = X/2$ , where  $Y$  is the dry soft parts weight,  $X$  is the shell cavity volume and  $Z$  is the dry shell weight, shell cavity volumes of: 2, 4 and 8 cm<sup>3</sup> give rise to respective dry soft parts weight of: 3, 5 and 9 g and Walne's CIs of: 3, 2.5 and 2.25. Even for the hypothetical case of parallel slopes (e.g., replacing the second equation  $Z = X/2$  by  $Z = X - 1$ ), CIs are: 3, 1.66 and 1.29. Because the previous example demonstrates that the variable of interest can be masked in the CI by the influence of the fixed variable, it seems to be more reliable to apply the approach (1) to monitor the reproductive cycle.

Advantages of the approach applied here based in the sequential mean drained soft parts weights are: (1) A common range for the initial data. Grant and Tyler (1983) affirmed that immature invertebrates should not be included in the analysis of gonad index,

as they will have smaller gonads than mature animals and their gonad weights, oocyte sizes, etc., are unlikely to follow the adult reproductive cycle. In consequence, the lower limit of our interval of shell lengths was restricted to 65 mm as the smaller specimens were not guaranteed to be mature animals. Alternatively, the upper limit of the interval was established in the maximal shell length of the samples. (2) Subsampled data were balanced by simulation and sequential sample means were estimated from a fixed set of values obtained from the arithmetic progression of shell lengths. Therefore, the final data was equally balanced with respect to the size of the specimens. (3) The standardization was unbiased because it was based on sample means. (4) The present biometrical approach involves only one variable (i.e., total drained weight), avoiding CIs. Dry soft parts weight or drained soft parts weight were probably more precise elections. However, we opted to look for the fastest approach, which reflected a reliable evolution of the reproductive cycle.

Accordingly, growth trends and maximums in mean total drained weight comprised between April and June were coincident with Phases III, followed by the falls and growths between June and August of Phases 0. Phases I started when maximums between August and October were reached and Phases II a month later. Finally, minimums between April and March matched the beginning of Phases III.

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## PARASITES OF THE STOUT RAZOR CLAM *TAGELUS PLEBEIUS* (PSAMMOBIIDAE) FROM THE SOUTHWESTERN ATLANTIC OCEAN

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**ABSTRACT** This work describes the parasites and their respective pathologies in the stout razor clam, *Tagelus plebeius* (Lightfoot, 1786) (Psammobiidae) from Argentinean coasts. It represents the first report about a histopathological survey for these species in the Southwestern Atlantic. Specimens of *T. plebeius* were collected at Mar Chiquita coastal lagoon (37°46'S, 57°27'W) ( $n = 104$  dissected under stereomicroscope,  $n = 28$  histologically sectioned and microscopically examined) and from the mouth of the Quequén Salado River (38°56'S, 60°33'W) ( $n = 14$  dissected under stereomicroscope). *Tagelus plebeius* was found housing two ciliate species without apparent host reaction and acting as first intermediate host for two digenetic trematode species of the families Fellodistomidae and Gymnophallidae, by hosting sporocysts (mainly in gonad and digestive gland that results in the replacement of host tissues) and as second intermediate host by housing a gymnophallid metacercaria. Likewise, *T. plebeius* was found parasitized by a larval spirurinae nematode encapsulated by hemocytes. The finding of two ciliate species, the gymnophallid cercaria and metacercaria, and the larval nematode represents the first record for the host. The Southwestern Atlantic populations of this clam seem to be devoid of serious pathogens in the study area.

**KEY WORDS:** *Tagelus plebeius*, pathology, parasites, southwestern Atlantic

### INTRODUCTION

The stout razor clam *Tagelus plebeius* (Psammobiidae) inhabits estuarine tidal flats along the American Atlantic coast from Cape Cod, Massachusetts (42°N, USA) (Leal 2002) to the north of Argentinean Patagonia (San Matías Gulf, 41°S) (Scarabino 1977). Despite its wide geographic distribution and its importance as dominant species in intertidal communities of some Southwestern Atlantic estuarine areas, to date a histopathological survey of this clam has not been undertaken. At present, only three parasites have been recorded: a protozoan of the genus *Perkinsus* (Dungan et al. 2002), a digenean larva at sporocyst stage containing trichocercous cercariae (Wardle 1983) and a cestode larva (Holland & Dean 1977). These reports are all from the Northern Hemisphere. Along the Argentinean coast, *T. plebeius* supports a small-scale artisanal fishery. Furthermore, histopathological surveys in commercially exploited bivalves are very scarce in Southwestern Atlantic coast (e.g., Cremonte & Figueras 2004, Cremonte et al. 2005).

The aim of this work is to report the parasites and the histopathologies that they evoke in the stout razor clam, *Tagelus plebeius*, from the Argentinean coast.

### MATERIAL AND METHODS

Specimens of *Tagelus plebeius* (Lightfoot, 1786) (Psammobiidae) ( $n = 104$ ) were collected from May 1996 to July 1997 at the intertidal of Mar Chiquita coastal lagoon (37°46'S, 57°27'W), Argentina by excavating the sediment with a shovel. Specimens measured 11–62 mm in maximum shell length (mean = 39). In January 1997, an additional sample of 14 specimens was collected at the mouth of Quequén Salado River (38°56'S, 60°33'W) comprising specimens of 31–68 mm in maximum shell length (mean = 53). Clams were examined for parasites under a stereomicroscope immediately after collection or fixed in 10% formaline to be

examined later. Digenean larvae were studied from whole aceto-carmin stained worms. Nematodes found both free and recovered from their capsules, were stored in 70% ethanol, cleared in lactofenol or alcohol-glicerine and examined under a light microscope. Drawings were made with the aid of a camera lucida. All larval measurements are given in micrometers as mean values followed by the range within parentheses. Ten digenean metacercariae and five nematode larvae were dried using the critical point method, examined with a scanning electron microscope (SEM) (Jeol/SET 100ti) and photographed. Prevalence (P) and mean intensity (MI) were calculated according to Bush et al. (1997). In June 2001, the soft parts of 28 clams from Mar Chiquita coastal lagoon, measuring from 26–67 mm of maximum shell length (mean = 55) were fixed in Davidson's solution (Shaw & Battle 1957) for 24 h and stored in 70% ethanol for histopathological survey. Tissue samples were embedded in Paraplast, and oblique transverse sections, approximately 5-mm thick, were taken from each specimen including mantle, gills, gonad, digestive gland, nephridia and foot. They were stained with haematoxylin and eosin. Histological sections were examined by light microscope under  $\times 400$  magnification for presence of parasites and pathological alterations.

To estimate the total number of metacercariae present, the cluster of larvae was detached, divided in four equal parts, the larvae present in one quarter counted and multiplied by four. The intensity of color and the calcareous alterations of the inner surface of shells were ranked in four categories as follows: 0 = no alteration, 1 = only dorsal area of shell slightly colored, 2 = dorsal area of shell heavily colored and extended to pallial sinus and 3 = almost the entire inner shell surface heavily colored with calcareous alterations present. The maximum shell length, the number of metacercariae and the intensity of shell alterations (using the 4 above referred categories) were correlated by a Spearman-rank test (Morales & Pino 1987).

Histological sections of parasitized clams were deposited at the Helminthological and Protozoological Collections, Museo de La

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Plata (MLP No. 5624, 5624/1, 5624/2 and MLP 023, 023/1, and 023/2). La Plata, Argentina; some valves of the examined clams were also deposited at the Malacological Collection, Museo de La Plata (MLP No. 5696).

## RESULTS

A total of six parasitic or commensal taxa were found in the clam samples examined; two of them were ciliate protozoans and the other four, metazoan parasites. Prevalences and mean intensities of infection found at both study sites are given in Table 1.

All measurements in this section will be assumed to indicate microns.

### *Ciliate sp. 1*

This protozoan was found in clams examined alive and from histological sections, mostly parasitizing the base of the gills in low intensities of infection (Table 1, Fig. 1 [2]).

### *Ciliate sp. 2*

This ciliate, which is similar to a *Trichodina* species, was also found in clams examined both alive and from histological sections parasitizing the gills in low intensities (Table 1).

### *Fellodistomid Cercaria sp.*

#### General Morphology

According to Cable (1956), a fellodistomid cercariae can be identified by the following characteristics: Distome, pharyngeate larva, tegument spinose or smooth, eyespots present or absent, stylet lacking, excretory vesicle thin-walled, and a long and slender trichocercous tail, developing in marine bivalves.

*Sporocyst* [measurements based on 10 mounted specimens]

Elongated, thick-walled sac without constrictions, 2,520 (1,750–3,051) in length by 310 (260–370) in maximum wide, each containing 10–21 (mean: 14) trichocercous cercariae at different developmental stages (Fig. 1 [3, 4]).

*Cercaria* [not released, measurements based on the 10 largest mounted specimens]

Body oval to pyriform, 343 (328–362) long by 154 (123–175) wide. Tegument spinose. Oral sucker subterminal. 65 (58–71) long by 67 (62–73) wide. Forebody (distance from anterior end of body to anterior edge of ventral sucker) 127 (120–135). Ventral sucker 52 (47–56) long by 51 (46–54) wide. Sucker ratio (ventral sucker length/oral sucker length): 1: 0.80 (0.79–0.81). Prepharynx absent.

Pharynx ovoid, 43 (39–52) long by 33 (31–37) wide. Esophagus 23 (17–30) long. Caeca bifurcating just anteriorly to ventral sucker. 151 (140–160) long by 18 (16–21) wide. Parenchyma full of cystogenous cells. Excretory vesicle V-shaped, arms reaching acetabular level; caudal excretory tubule conspicuous, opening at end of tail in two pores. Primordial testes opposite, located at the level of the caecal end. Tail 484 (452–520) long by 47 (43–51) wide, with about 25 finlet-like structures on each side with 15 (9–22) setae joined by a membrane (Fig. 1 [5]).

#### Site of Infection

Mainly in gonad, also in digestive gland.

#### Histopathology

Sporocysts were replacing host tissues (Fig. 1 [3]).

### *Gymnophallid cercaria sp.*

#### General Morphology

According to Bartoli (1974), a gymnophallid cercaria can be identified by the following characteristics: Distome, pharyngeate larva, tegument spinose, eyespots absent, stylet lacking, excretory vesicle thin-walled V or Y shaped, furcated tail, developing in marine bivalves.

*Sporocyst* [measurements based on 10 mounted specimens]

Elongated, thin-walled sac without constrictions, 750 (470–1101) long by 250 (160–331) in maximum wide, each containing 50–55 (mean: 50) furcocercariae at different developmental stages (Fig. 1 [6a]).

*Cercaria* [not released, measurements based on the 10 largest mounted specimens]

Body minute, transversely spinose, 136 (126–151) long by 61 (52–67) wide at ventral sucker level. Oral sucker opening subterminally, 35 (30–41) long by 31 (29–36) wide. Ventral sucker located in hindbody, 25 (23–28) in diameter. Sucker ratio (ventral sucker length/oral sucker length): 1:1.4 (0.68–0.77). Pharynx ovoid, 20 (16–23) long by 15 (12–17) wide. Esophagus 15 (13–16) length. Caeca short, reaching acetabular level. Excretory vesicle V-shaped filled with excretory granules, arms reaching pharynx level, opening on the inner side of each furca end. Tail stem 45 (41–51) in length, furcae 55 (50–65) in length (Fig. 1 [6b]).

TABLE 1.

Prevalence and mean intensities of parasites of the stout razor clam *Tagelus plebeius* from Argentina.

	Mar Chiquita Coastal Lagoon (n = 104)		Mar Chiquita Coastal Lagoon (n = 28)		Quequén Salado Mouth River (n = 14)	
Examination method	Dissection under stereomicroscope		Microscopical examination of histological sections		Dissection under stereomicroscope	
Prevalence (P) and mean intensity (MI)	P (%)	MI	P (%)	MI	P (%)	MI
Ciliate sp. 1	Not quantified		25	low	Not quantified	
Ciliate sp. 2 ( <i>Trichodina</i> sp.)	Not quantified		0.35	low	Not quantified	
Fellodistomid sporocysts	0.96	—	0	—	0	—
Gymnophallid sporocysts	0.96	—	0	—	7.14	—
Gymnophallid metacercariae	100	662	54	low	100	546
Spirurine larval nematodes	35.58	3.38	Not quantified	Not quantified	21.43	1.33



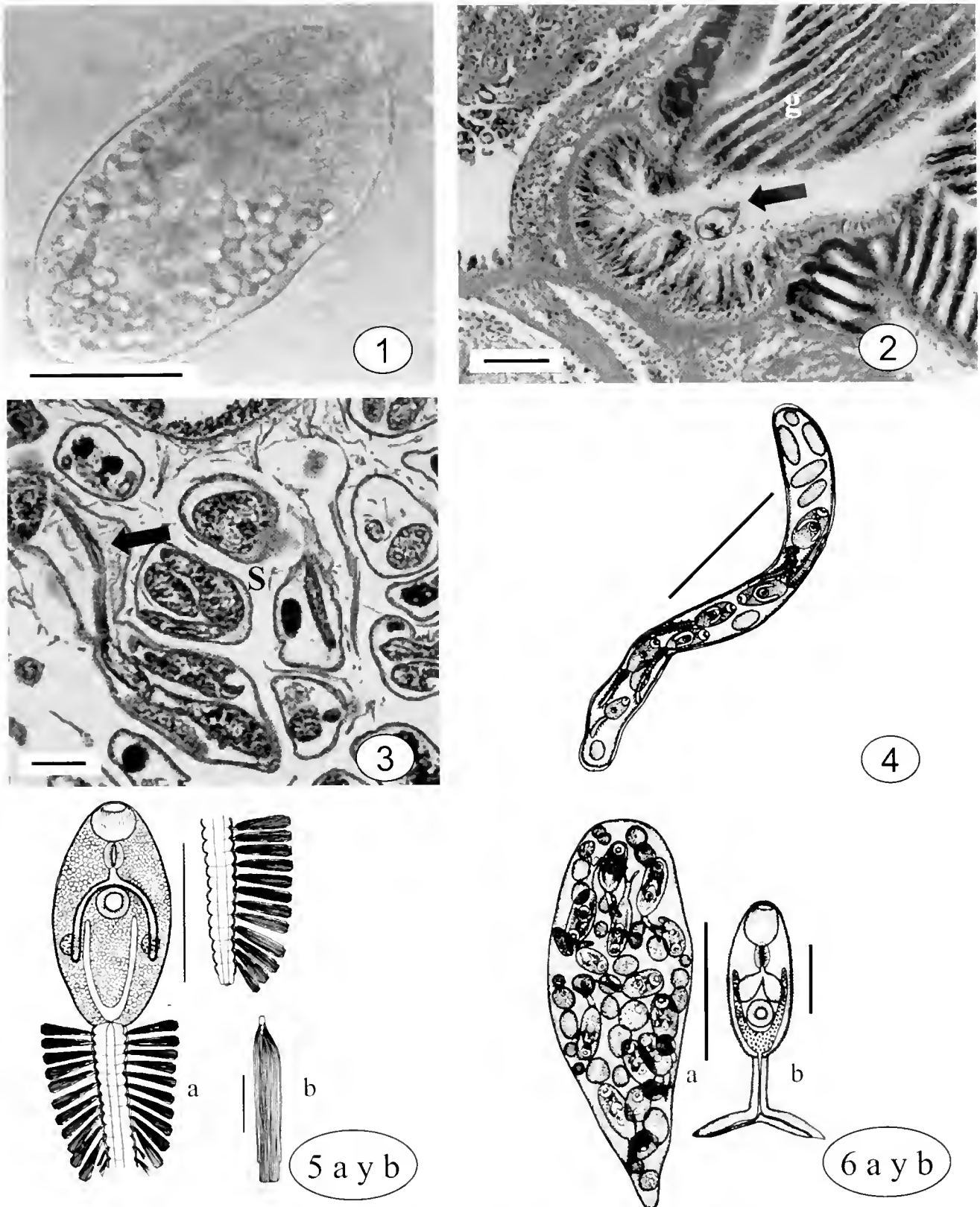


Figure 1. Parasites of *Tagelus plebeius* from Southwest Atlantic coast. 1. Live specimen of ciliate sp. from the gill, scale bar = 20 µm. 2. Histological section showing a ciliate at the base of gills, scale bar = 250 µm. References: g = gill. 3. Gonad replaced by the fellodistomid sporocysts, note the trichocercous tail of cercariae (arrow), scale bar = 600 µm. References: s = sporocyst. 4. Sporocyst of Fellodistomidae (Digenea), scale bar = 200 µm. 5a. Cercaria of Fellodistomidae, scale bar = 200 µm. b. Detail of a set of the trichocercous furcae, scale bar = 30 µm. 6a. Sporocyst of Gymnophallidae (Digenea), scale bar = 200 µm. 6b. Cercaria of the family Gymnophallidae, scale bar = 50 µm.

### Site of Infection

Mainly in gonad, also in digestive gland.

### Histopathology

Sporocysts were replacing host tissues.

### *Gymnophallid metacercaria sp.*

#### General Morphology

According to Bartoli (1974), a gymnophallid metacercariae can be identified by the following characteristics: small or minute worms, tegument spinose, oral sucker larger than ventral sucker with or without lateral projections, ventral sucker equatorial to postequatorial, excretory vesicle V or Y shaped, with long lateral arms extending to pharyngeal level and filled with excretory granules. Reproductive organs in an advanced stage of development (testes opposite to diagonal, ovary pre or post testicular).

*Metacercaria* [measurements based on 10 largest mounted specimens]

Body small, oval to pyriform, 329 (284–374) long by 167 (110–230) wide at ventral sucker level. Body rounded when enveloped by host tissues. Spines arranged transversely over all body length. Oral sucker subterminal, 70 (46–90) long by 75 (27–90) wide, with two conspicuous lateral projections. Twelve papillae located around oral sucker opening. At least 5 pairs of cephalic glands opening dorsally to oral sucker. Ventral sucker post equatorial, 35 (30–41) in diameter, with 6 papillae. Sucker ratio (ventral sucker length/oral sucker length): 1:0.46 (0.50–0.65). Pharynx ovoid, 34 (29–38) long by 29 (23–41) wide. Esophagus 19 (10–40) in length. Caeca 84 (63–98) long by 54 (37–88) wide, reaching acetabular level. Testes ovoid to rounded, located postero-lateral to ventral sucker, symmetrically to obliquely disposed. Testis posterior to ovary, 49 (37–60) long by 41 (32–58) wide; testis opposite to ovary 46 (28–62) long by 42 (34–47) wide. Ovary rounded, pretesticular 30 (28–32) in diameter. Genital pore wide, oval, located somewhat apart and anteriorly to ventral sucker. Genital atrium oval and shallow. Vitellaria formed by two compact lobes, located at sides of ventral sucker. Excretory vesicle V-shaped, with diverticulated arms reaching oral sucker and filled with excretory granules (Fig. 2 [7–10]).

### Site of Infection

Metacercariae lay forming a compact and easily detachable cluster (see Fig. 3 [13] later) just below the dorsal shell margin, immediately below hinge and above the rectum (Fig. 2 [11]). In heavy infections metacercariae were also found in the extrapallial space adjacent to the anterior end of the pallial sinus (Fig. 2 [11] Fig. 3 [13]).

### Histopathology

Macroscopically, metacercariae appear crowded, forming an orange-colored cluster enveloped by host tissues (Fig. 3 [13]). In heavy infections, a brownish-orange pigmentation is also present on the inner shell surface at the sites where metacercariae are located (Fig. 3 [12]) (i.e., below hinge) and also along the pallial sinus. In histological sections, each metacercaria appear surrounded by a hyaline, non cellular envelope, which is formed by several concentric layers (Fig. 3 [14]). This envelope has a jelly-like appearance under the stereomicroscope (Fig. 3 [13]). Metacercariae and their envelope are surrounded, individually or in groups, by a sac formed by one-cell-thick cubic or somewhat

flattened epithelium (Fig. 3 [14]), which results from the invagination of the outer mantle epithelium at the isthmus region. Cumuli of orange pigment were observed as amorphous aggregates, between the epithelial layer and the hyaline envelope of each metacercaria (Fig. 3 [14]). In some clams, abnormal calcifications in the form of loose calcium concretions or growth disruptions on the inner shell surface were present, which were from slightly to strongly colored (Fig. 3 [12]). Small calcareous concretions like pearls or amorphous calcium carbonate were also found within sacs, near dead metacercariae (Fig. 3 [12–14]).

### Relationship Between Shell Length, Shell Alteration and Intensity of Infection by *Gymnophallid metacercariae*

Spearman-rank correlation test was statistically significant in all cases ( $n = 104$ ;  $P < 0.001$ ),  $r_s = 0.85$  obtained when plotting maximum shell length and intensity of infection by metacercariae;  $r_s = 0.45$  obtained when plotting maximum shell length and shell alteration;  $r_s = 0.45$  obtained when plotting intensity of infection by metacercariae and shell alteration.

### *Spirurina larval nematode*

#### General Morphology

According to Chaubaud (1975), a nematode with the following characteristics can be included into the Order Spirurida Chaubaud, 1975, Suborder Spirurina Chaubaud, 1974: anterior extremity bilaterally symmetrical, pseudolabia well developed, esophagus divided into an anterior muscular portion and a posterior, longer and glandular portion.

*Description* [measurements based on 20 specimens]

Stout small nematode with cuticle finely and transversely striated and pseudolabia well developed. The lateral groove starts in the anterior part and ends near fasmids. Two cephalic papillae are at each side, and amphids large with Deirids absent. Body unarmed, 1,238 (805–1,610) long by 47 (32–68) wide. Esophagus elongate, clavate, 278 (102–410) long; divided into anterior muscular part, 116 (88–149) long, and posterior glandular part, 177 (109–272) long. Nerve ring slightly anterior to the junction of both parts of esophagus, distant 106 (91–123) from anterior end. Excretory pore 185 (129–223) from anterior end. Genital primordia observed in some specimens as one cell located in the middle of body length. Three rectal cells (one dorsal and two ventral). Tail conical, 96 (81–120) long, ending in a sharp cuticular spike (Fig. 4 [15–19]).

### Site of Infection

Muscular wall of visceral mass (48%), labial palps (28%), siphon retractor muscles (8%), adductor muscles (8%), radial muscles of the mantle border (4%), mantle (4%) (Fig. 5 [20–23]).

### Histopathology

Larvae are found free or individually surrounded by a capsule. At the stereomicroscope, the capsule appears as a brownish spot that measures 1–3 mm in diameter; larva inside is alive and able to move. The degree of tissue reaction elicited by the presence of this parasite varies from no reaction to the formation of a thick capsule (Fig. 5 [20–23]). Histological sections show that the capsule is formed by a dense aggregation of hemocytes (Fig. 5 [22, 23]). In some cases, bundles of fibers of the muscle affected are seques-

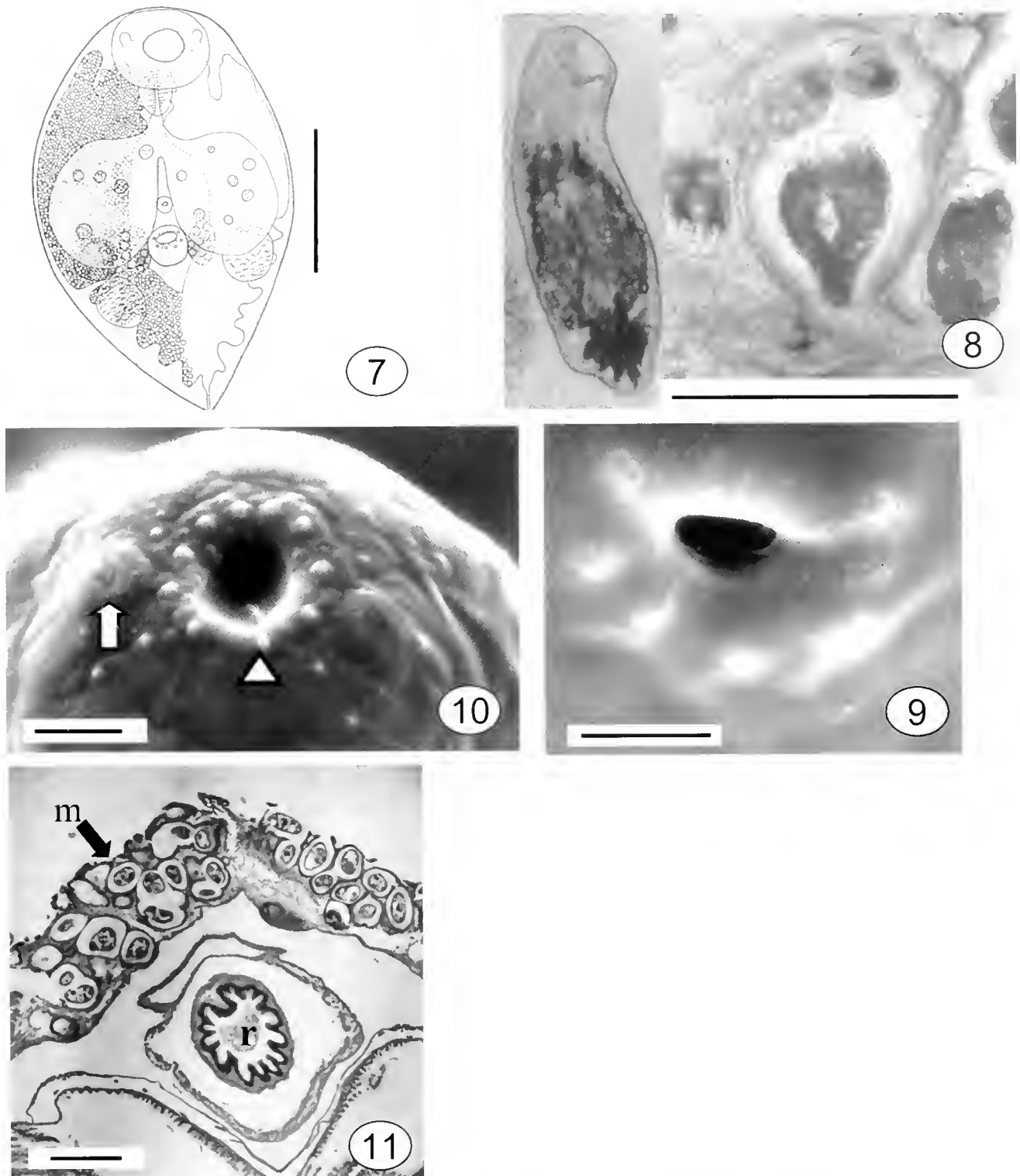


Figure 2. Parasites of *Tagelus plebeius* from Southwest Atlantic coast. 7. Metacercariae of Gymnophallidae, scale bar = 100 µm. 8. Live specimens of metacercariae of the family Gymnophallidae, scale bar = 300 µm. 9. Detail of oral sucker at SEM, note the two lateral projections slightly retracted (arrow) and papillae (arrow head), scale bar = 10 µm. 10. Detail of ventral sucker at SEM, note the six papillae, scale bar = 10 µm. 11. Histological section of dorsal part where gymnophallid metacercariae are located scale bar = 200 µm. References: m = metacercaria, r = rectum.

tered to form part of the outer wall of the capsule (Fig. 5 [23]). In other cases, the reaction complex only causes the splitting of the adjacent muscle fibers; however, the later do not form part of the capsule.

#### DISCUSSION

The present report is the first parasitological survey of the stout razor clam *Tagelus plebeius* from the Southwestern Atlantic

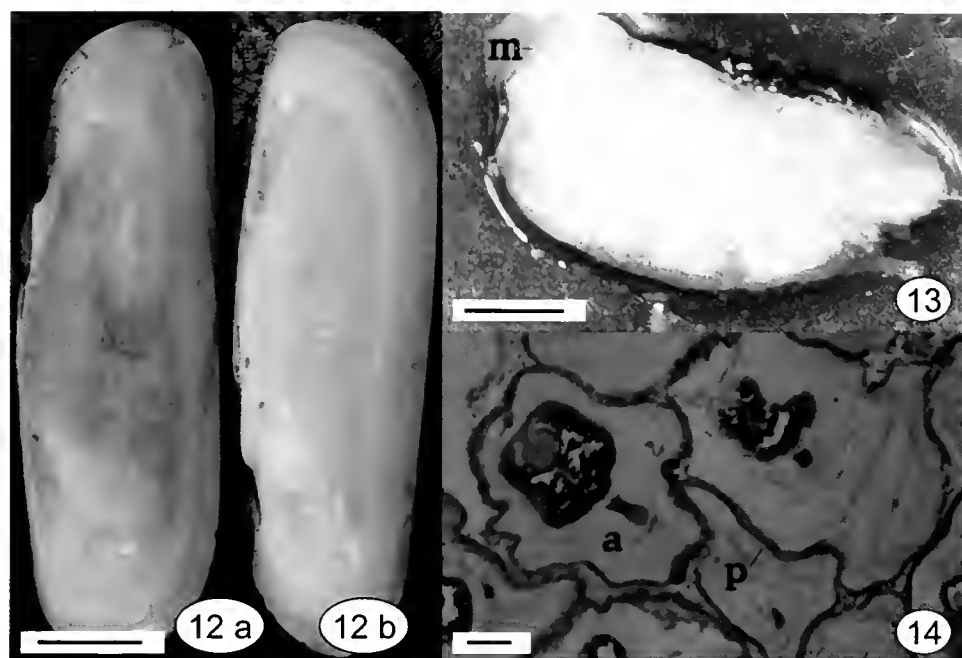


Figure 3. Shell alterations in *Tagelus plebeius* caused by gymnophallid metacercariae. 12a. Left valve strongly colored in a specimen with high parasitic infection by gymnophallid metacercariae; 12b. Left valve slightly colored in a specimen with light parasitic infection of gymnophallid metacercaria, scale bar = 2 cm. 13. Metacercaria crowd removed from a hivalve specimen, scale bar 1000  $\mu$ m. 14. Histological section of *Tagelus plebeius* showing the pigment accumulated inside the sacs, scale bar = 200  $\mu$ m. References : m = metacercaria; a = noncellular matrix; p = orange pigment; e = epithelium.

Ocean. The finding of two ciliate species, the gymnophallid cercaria and metacercaria, and the larval nematode represent first records for the host. The only digenean previously recorded in *T. plebeius* is a fellodistomid cercaria (Wardle, 1983). The larval cestode (Holland & Dean 1977) and the protozoa species of *Perkinsus* (Dungan et al. 2002), all reported from the Northwestern Atlantic coast were not found in this study. From biogeography point of view, this result is in accordance with that hypothesized for many adult parasites: most parasite species have restricted, continental, geographical distributions, even though their hosts have wider, intercontinental, distributions (Carney & Dick 2000).

*Tagelus plebeius* was found acting as first intermediate host for two digenetic trematode species of the families Fellodistomidae and Gymnophallidae, hosting the sporocyst stage. Most trematode species parasitize gastropods. Only a few families infect bivalves, and as were found in this study, low prevalences were the rule (Lauckner 1983). In our study, sporocysts of both families were found replacing host tissues without host reaction. They do not become encapsulated when they are found in their natural intramolluscan habitats because both are well adapted with each other (Cheng & Rifkin 1970).

According to Bray (1988), nonoculate cercariae carrying long trichocercous tails without furcae, with fin-like setae joined by a membrane and spinose tegument belong to the subfamily Baccigerinae Yamaguti, 1958. Thus, the fellodistomid cercaria observed in the present study likely belongs to the subfamily Baccigerinae. The life cycle of Baccigerinae involves a heterodont bivalve, which acts as first intermediate host, some representative of gelatinous plankton or a crustacean usually act as second intermediate hosts and fishes are the definitive host (Bray 1988).

As regard the finding of cercaria of the family Gymnophallidae, its identification below family or subfamily level is not possible

because cercariae of different species in this family are almost indistinguishable (Lauckner 1983). Because most gymnophallid life cycles have a swimming cercaria that enter in a second intermediate host that is the same species as the first one, it seems probable that gymnophallid cercaria found belongs to the same species that the metacercaria hosted by *T. plebeius* (Cremonte 2004). It would be necessary to perform experimental infections to confirm this assumption and to obtain the adult form to identify it at genus and species level. High prevalences and intensities of gymnophallid metacercariae (Table 1) could be explained by the hydrodynamic of the studied environment. The quiet circulation of water masses facilitates the transmission of cercaria (Bartoli 1984). The oystercatcher, *Haematopus palliatus* (Aves: Haematopodidae) seems to be the definitive host, because it was reported to feed mainly on *T. plebeius* along this environment (Bachmann 1995).

The only gymnophallids reported from South American mollusks are *Lacunovermis* sp. from *Patinigera* spp. (Gastropoda: Patellidae) (Martorelli & Morriconi 1998), *Bartolius* sp. from *Gaimardia trapesina* (Bivalvia: Gaimardiidae) (Ituarte et al. 2001) (both from Magellan Strait and Beagle Channel) and *Bartolius pierrei* from *Darina solenoides* (Bivalvia: Mactridae) from Patagonian coast (Cremonte 2001).

Regarding the pathology caused by metacercariae of the family Gymnophallidae, it is variable and depends on the parasite and the host species involved (e.g., deposition of additional shell material in the form of calcareous concretions, blisters and crests, ridges, or igloo-like structures on the inner surface of the valves, pearls, shell erosions like pits, depletion of host body reserves, alterations of host behavior and general debilitation and morbidity) (Lauckner 1983, Ituarte et al. 2001, Cremonte & Ituarte 2003, Ituarte et al. 2005). In the present case, the host reaction observed in *T. plebeius* is very similar to that reported in *D. solenoides* infected by *B.*

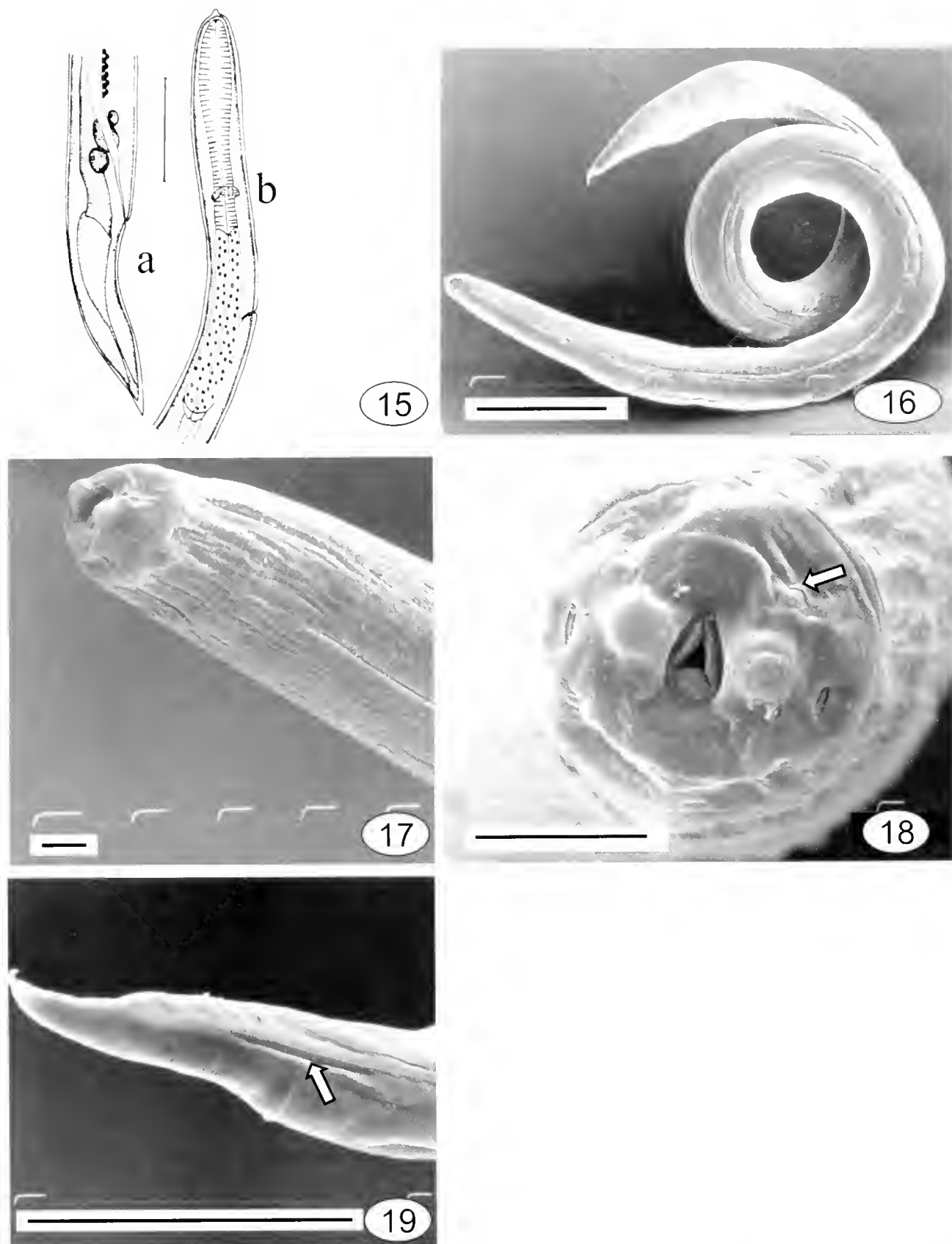


Figure 4. Larval nematode of the subfamily Spirurinae. 15a. posterior extremity; 15b. anterior extremity, scale bar = 50 µm (16–19). SEM photographs of the larval spirurine nematode in *Tagelus plebeius* from Argentina. 16. Whole coiled worm, scale bar = 100 µm. 17. Anterior part of the body, note the lateral groove and the absence of deirids, scale bar = 10 µm. 18. Apical view of the anterior end, showing pseudolabia, cephalic papillae (arrow) and amphids, scale bar = 10 µm. 19. Caudal end, note the terminal cuticular spike, anus and the end of the lateral groove (arrow), scale bar = 10 µm.

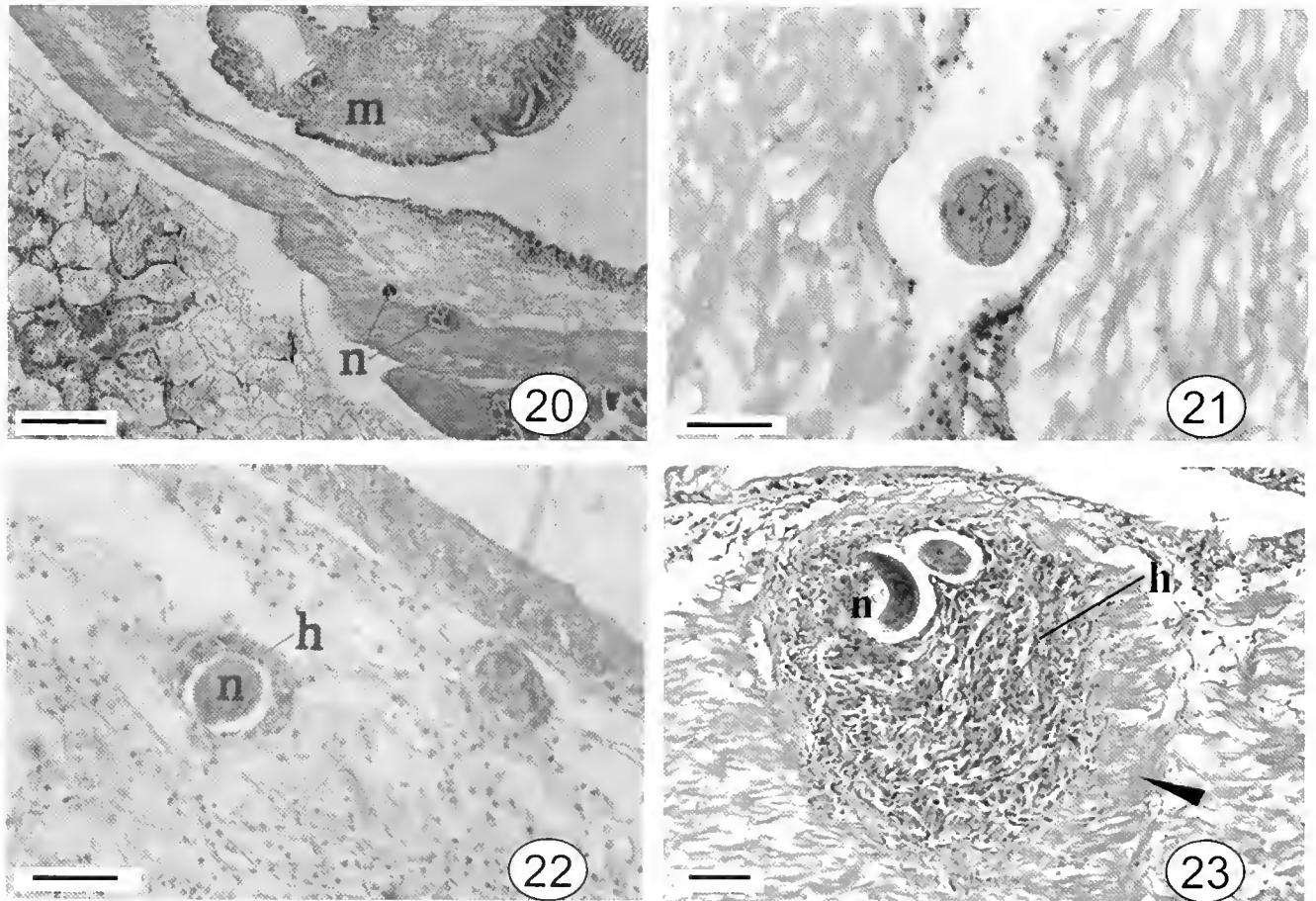


Figure 5. Histological section of *Tagelus plebeius* parasitized with larval spirurine nematode from Argentina. 20. General view showing larval nematode transversally sectioned in connective tissue of mantle and muscle, scale bar = 200  $\mu$ m. 21. Detail of larval nematode transversally sectioned at esophagus level showing muscle fibers divided, note hemocytes, scale bar = 50  $\mu$ m. 22. Initial formation of capsule by hemocytes (h) in connective tissue of mantle, scale bar = 50  $\mu$ m. 23. Advanced stage of a capsule formed by hemocytes in tegument musculature (arrow), scale bar = 100  $\mu$ m. References: n = nematode; m = mantle; h = hemocytes.

*pierrei* (Cremonte & Ituarte 2003). The main difference observed in *T. plebeius* is the presence of a brownish-orange colored pigment within sacs containing larvae, particularly in older bivalve hosts, in concordance with correlations among shell length, shell alteration and intensity of infection observed. A similar but yellowish conchiolin material was reported in *Tellina* spp. (Bivalvia: Tellinidae) by Giard (1897) and brownish material by Bartoli (1974) in *Tapes aureus* (Bivalvia: Veneridae) infected by gymnophallid metacercariae.

Metacercariae of Gymnophallidae are known to be able to prevent naereazation around them, allowing most larvae to remain alive inside their envelope until reaching a suitable definitive host. This peculiarity of the gymnophallids encapsulation was discussed by Ituarte et al. (2001), Cremonte & Ituarte (2003) and Ituarte et al. (2005). However, in advanced stages of parasitism (i.e., in older infections) it seems that several metacercariae die, and just after this, the deposition of calcium to form blisters seems to be possible. In *T. plebeius*, calcium concretions in the form of blisters or loose pearls were only seen in older clam specimens, and seem to affect a reduced number of adult bivalves. Lomovasky et al. (2005) reported this phenomenon in only the 5.9% of the 620 specimens of *T. plebeius* studied.

Marine bivalves, as a group, are rather uncommon hosts for

nematodes. However, ascaridoids and gnathostomids have been reported several times from representatives of commercially exploited bivalve species (Lauckner 1983). The nematode taxa more frequently reported from marine bivalves are *Sulcascaris sulcata* (Ascaridoidea: Anisakidae) and *Echinocephalus* spp. (Gnathostomoidea: Gnathostomidae); their adults occur in fishes, which prey on clams (Sindermann 1990). The nematode larva described in this study clearly does not correspond to an ascaridoid, because it has pseudolabia and an esophagus divided into glandular and muscular portions (Anderson et al. 1974). Moreover, the larva described here is not a gnathostomid because it has not trilobed pseudolabia and lacks the anterior extremity swollen into bulb (Anderson et al. 1974). Thus, the present record represents a new group of nematode using marine bivalves, *T. plebeius* in this case, as intermediate or paratenic hosts.

High values of prevalence and mean intensity of infection found in this study (Table 1) indicate these are not cases of an accidental infection. This represents the first record of a nematode larva parasitizing a bivalve in the Southwestern Atlantic Ocean. Because all nematodes found in marine bivalves are larvae, they are extremely difficult to identify (i.e., lack the diagnostic characters that are carried by the adult stage); thus, the importance of nematodes as parasites of this group have been sadly neglected



(Cheng 1978). The nematode larva described in this survey is not specific regarding its site of infection, being located mainly in muscles but also in other organs such as labial palps. Sites of infection of larval nematodes in other bivalves also showed to be highly variable: *Echinocephalus uncinatus* Molin, 1858 was found in the adductor muscle of pearl oyster *Margaritifera vulgaris*, *E. pseudouncinatus*, Millemann, 1951 in the foot of pink abalone *Haliotis corrugata* (Millemann 1963) and *E. crassostreai* Cheng 1975 in the gonoducts of Japanese oyster *Crassostrea gigas* with a reaction of the tunic elements that are surrounding the gonoduct (Cheng 1975).

The pathogenicity of larval nematodes in marine bivalves varies from nonreaction to a strong tissue response (Ko et al. 1975). Different degrees of host reaction observed in the present work would correspond to the time of infection. The capsule formed by a dense aggregation of hemocytes such as observed in *T. plebeius*,

is known as hemocytosis according to Cheng & Rifkin (1970). Despite the large number of capsules studied, fibrous-appearing nodules as it was described by Harris (1975) for the case of *Angiostrongylus cantonensis* (Metastrongylidae) parasitizing a gastropod, were not observed. Harris (1975) noted some variability in the extent of response even within the same snail host. In that case, the granuloma loses the appearance of a loose aggregation of basophilic cells and becomes more fibrous and eosinophilic, similar to the capsule reported for the cestode *Echinobothrium* sp. by Cheng (1978).

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## COMPARISON OF ASSIMILATION EFFICIENCY ON DIETS OF NINE PHYTOPLANKTON SPECIES OF THE GREENSHELL MUSSEL *PERNA CANALICULUS*

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**ABSTRACT** The greenshell mussel *Perna canaliculus* is the most important species in aquaculture in New Zealand. Mussel energetics and growth rates are subject to the natural variability in phytoplankton biomass and species composition and thus understanding the influence of food type on assimilation efficiency is fundamental to the prediction of mussel production and planning farm management. In this study pulse-chase feeding techniques were used to assess the effect of diet on assimilation efficiencies for nine phytoplankton species including three diatoms *Chaetoceros calcitrans*, *Skeletonema costatum* and *Thalassiosira* sp., and three flagellates *Eutreptiella* sp., *Pyrammonas* sp. and *Isochrysis galbana*, and three dinoflagellates *Akashiwo sanguineum*, *Alexandrium minutum* and *Gymnodinium catenatum*. Assimilation efficiency varied with algal species, but it was significantly higher when mussels were fed dinoflagellates (84.5%) compared with diatoms (61.7%) and flagellates (77.9%). Assimilation efficiency of dinoflagellates and flagellates increased with gut passage time, whereas with a diatom diet, a negative correlation was evident. This finding has implications in understanding and predicting growth rates of mussels (and hence commercial yield) in tandem with natural variability in phytoplankton species composition.

**KEY WORDS:** mussel, *Perna canaliculus*, phytoplankton, assimilation efficiency, gut passage time

### INTRODUCTION

The feeding responses of bivalves to variations in the quantity and quality of seston have been extensively studied (see reviews by Winter 1978 and Jørgensen 1996). Results of these studies have shown the ability of bivalves to preferentially ingest organic particles from the filtered matter and selectively reject the inorganic particles. Many of these feeding experiments have been conducted on blue mussels (e.g., Bayne et al. 1989, Bayne et al. 1993, Navarro et al. 1992), oysters (e.g., Newell & Jordan 1983, Barillé et al. 1997, Ren et al. 2000), scallops (e.g., Cranford et al. 1998), clams (e.g., Hartwell et al. 1991), and cockles (Iglesias et al. 1992 & Iglesias et al. 1996). Few studies used *P. canaliculus*, the greenshell mussel (Hawkins et al. 1999). In these studies, diets of either natural seston or natural food with addition of algae were used to understand the effects of food quantity and quality on feeding dynamics. Seston-phytoplankton mixed diets generate a blended assimilation that reflects a diversity of particle sources and quality. To our knowledge however, relatively little attention has been paid to the preferential ingestion among phytoplankton species (e.g., Romberger & Epifanio 1981, Shumway et al. 1985).

Considerable intra-annual variations in growth rates and conditions of farmed shellfishes have been observed in Marlborough Sounds, New Zealand (e.g., Ross et al. 1997, Ross & Image 2001). This reflects the complex ecophysiological response of mussels to the equal complexity of interactions among environmental variables. High phytoplankton biomass usually results in fast growth and increase in condition of mussels (Ren & Ross 2005). Chlorophyll-*a* pigment concentration is often used to estimate phytoplankton biomass in field monitoring and, therefore, can be an indicator of environmental conditions that influence mussel growth. Over the last few years, the chlorophyll-*a* concentration in Marlborough Sounds has generally decreased and there have been concurrent decreases in both the condition and growth rate of the mussels (e.g., Ross et al. 1997, Ross & Image 2001). Although these trends appear to be broadly related, there have been occa-

sions when mussel growth has been poor even though surrounding chlorophyll-*a* levels have been moderate (Ren & Ross 2002). These discrepancies may relate to the differential consumption of phytoplankton species or groups. During the same period, the composition and abundance of the phytoplankton was characterized by strong intra and interannual variations in the extensive mussel farming area of Marlborough Sounds. This variation may even be pronounced during seasons of algal blooms when one single species usually dominates the phytoplankton biomass (Mackenzie et al. 1986, Ogilvie 2000). This raises the question on whether mussels can assimilate some algal species with greater efficiency than others and hereby show good growth even when chlorophyll-*a* as an indicator of total phytoplankton biomass is low. Selective ingestion on different algal species has been observed in other mussel species (e.g., Kreeger et al. 1996, Wang & Fisher 1996). The mussel *Mytilus galloprovincialis* preferentially selects dinoflagellates rather than diatoms and digestion rate of the dinoflagellates may also vary among species (Sidari et al. 1998). Although a few studies on feeding behavior of *P. canaliculus* have been done (Hawkins et al. 1999), very little is known of the selective assimilation in this species. To assess energy flows through a mussel-farmed ecosystem it is essential to quantify the efficiency with which the mussels assimilate the phytoplankton species and groups that inhabit the Marlborough Sounds.

Assimilation efficiency is usually estimated from direct measurements based on the ash ratio between the food source and feces as developed by Conover (1966). This ratio is embodied in the mass balance equation that assesses scope for growth, which is based on measured difference between energy gains from feeding and energy loss for respiration and excretion (Baynes & Newell 1983). However, the relationship between estimated growth from mass balance models and actual growth has not been convincingly described in bivalves (e.g., Riisgård & Randol 1981). By comparing estimated and actual growth of mussels, the authors found that estimation of assimilation efficiency gave unreliable results. Although uncertainty in modeling techniques may not be ruled out (see van Haren & Kooijman 1993), limitations of the methods used in physiological measurements may, to some extent, have contributed to the error of estimated assimilation efficiency. As discussed by Conover (1966), when the diet consists of a low ratio of ash to

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organic content such as phytoplankton, a significant proportion of ash can be absorbed. This would result in uncertainties in estimated assimilation under such a condition. This method also requires a large amount of material and is susceptible to weighing errors even when using a microbalance (Navarro & Thompson 1994). In shellfish farming ecosystems such as Marlborough Sounds of New Zealand, the contribution of phytoplankton biomass to total organic particulate matter is usually very low in terms of dry weight, but it is the main energy sources for bivalve growth. Small errors in measurements can potentially result in considerable larger errors in estimation of energy budgets (Ren & Ross 2005). Therefore, accuracy in estimates of the bioenergetic parameter is critical for the study of mussel energetics.

In this study, we used a radiotracer technique to investigate how mussels respond to different food diets. Assimilation efficiencies were measured in *P. canaliculus* using nine phytoplankton species found in a mussel-farming ecosystem in Marlborough Sounds, New Zealand. This study is aimed at providing information for parameterizations of energetic and carrying capacity models currently being developed (Ren & Ross 2005).

## MATERIALS AND METHODS

### Mussels

Three hundred mussels (*P. canaliculus*, ~70 cm shell length) were collected on one occasion from a farming site in Marlborough Sounds, New Zealand. They were transported immediately to and kept on ropes in Magazine Bay near Christchurch for use in the experiments. For the feeding trial, 54 mussels were transferred to the NIWA laboratory in Christchurch and acclimatized in a recirculating aerated flow-through seawater system for 72 h prior to feeding experiments. During the acclimatization period, mussels were fed with a diet of mixed algae consisting of nine experimental algae with equal numbers of each species. Approximately  $1 \times 10^9$  cells of the algae were added each day per mussel to ensure that physiological activities of the animals were not affected because of starvation. Seawater was collected directly from Christchurch coast and stored in three holding tanks. Because the seawater was kept in the dark and aerated in the laboratory for over 6 wks before experiments, there were no living algae in the water at the time of use. Seston, particulate organic matter and chlorophyll-*a* concentrations were  $2.15 \text{ mg L}^{-1}$ ,  $0.49 \text{ mg L}^{-1}$  and  $\sim 0 \text{ } \mu\text{g L}^{-1}$  respectively. Temperature was constant at  $\sim 16^\circ\text{C}$ . The seawater was recirculated and monitored once a day for ammonia concentration using ammonia test kits. Throughout all experiments, the ammonia concentration never exceeded 1 ppm.

### Experimental Diets

An important aspect of this study was to measure assimilation efficiency that can be incorporated into energetic and carrying capacity models applied in mussel farming ecosystems. It is therefore important that these measurements be conducted on diets reflecting natural conditions. In mussel farming sites of the Marlborough Sounds, there is little variation in POM, whereas there is a considerable inter and intra annual variation in phytoplankton species composition. Phytoplankton contributes very little to POM in terms of dry weight (<10%, e.g., Ren 2001), but it is the major food sources for bivalve growth in terms of available suspended particulates (e.g., James & Ross 1996, Ogilvie 2000). Therefore, to

be representative of the natural condition, experimental diets were based on  $^{14}\text{C}$  labeled algae.

Nine phytoplankton species, *Chaetoceros calcitrans*, *Skeletonema costatum*, *Thalassiosira* sp., *Akashiwo sanguineum*, *Alexandrium minutum*, *Gymnodinium catenatum*, *Entreptiella* sp., *Isochrysis galbana*, *Pyramimonas* sp., belonged to diatom, flagellate and dinoflagellate, were cultured at the NIWA aquaculture laboratory at Mahanga Bay. The algae were enumerated by particle counter (Model ZM and tube with  $100\text{-}\mu\text{m}$  orifice diameter). The algae were spiked with  $^{14}\text{C}$  ( $100 \text{ } \mu\text{Ci mL}^{-1}$ ) by exposing the algae to the radiotracers for 3 d and kept in 12:12 light-dark photoperiod at  $17^\circ\text{C}$ . To calculate  $^{14}\text{C}$ -uptake after 3 d exposure, 2 mL replicate samples were each filtered through a 25 mm diameter GF/F filters ( $0.7\text{-}\mu\text{m}$  pore, Whatman). These filters were then added to 1 mL 5% HCl and held for over 6 h for liquid scintillation cocktail for analysis (LKB Wallac 1217 Rackbeta) of  $^{14}\text{C}$ -activity. Before feeding to mussels, the algal cells were separated from their culture medium by centrifugation (1000 g, 10 min) to remove dissolved  $^{14}\text{C}$ . The algal pellets were rinsed with  $0.7\text{-}\mu\text{m}$  filtered seawater. This process was repeated three-times to remove surface attached chemicals. The cells were resuspended in 100 mL seawater and enumerated for the feeding experiments as described below.

### Experiments with Mussels

Radiolabeled algae were transferred into a 20-L container and mixed with 15 L seawater (no living algae). Nine experimental trials were conducted simultaneously and experimental diets were prepared by adding  $^{14}\text{C}$  labeled algae into seawater. Each experimental trial was identical except that mussels were fed on different  $^{14}\text{C}$  labeled algal species. Six replicate individual mussels were then placed in the container containing the mixture of seawater and radiolabeled algae ( $\sim 1 \times 10^5 \text{ cells mL}^{-1}$ ) for 30 min. The duration of the feeding was assumed sufficient to allow mussels to ingest a substantial amount of the labeled algae but not defecate, because the experimental duration was less than gut transition time of 80 min (Hawkins et al. 1999). The solution was stirred and aerated to homogenize algae during the course of the feeding trial. Pseudofeces production was not observed; this was probably caused by particulate concentrations of the diets being lower than the threshold for pseudofeces production. Feces were collected at the end of the feeding trial, but  $^{14}\text{C}$  activity in these were negligible.

After feeding with radiolabeled algae, the mussels were rinsed with seawater and transferred into a flow-through system to "chase" the unassimilated material through the gut. The mussels were fed with unlabeled mixed algal diet the same as prior to the experiment. In a separate pilot trial, gut evacuation time of the mussel varied with algal species, but most of ingested diets were evacuated within 30 h. In this experiment, mussels were allowed to evacuate their guts for 3 d. This was also based on evidence that *Mytilus edulis* completes its digestion and assimilation of food within 3 d (see Wang et al. 1995). Feces produced by individual mussels were collected with a wide-pore pipette to monitor gut passage of the radioactive algae every 1–3 h for the first 10 h and every 4–20 h thereafter. Feces were filtered onto 25 mm diameter GF/F filters ( $0.7 \text{ } \mu\text{m}$  pore, Whatman) and individually placed into 20 mL scintillation vials until analysis.

After a 3 d pulse-chase feeding experiment, ingested  $^{14}\text{C}$  algae were to have been assimilated and incorporated in the mussel flesh, which was then dissected from the shell, added to a tared aluminum weighing pan, freeze-dried and then reweighed to determine

total dry flesh weight of each mussel. Dried flesh of individual mussels was then ground with a mortar and pestle and three replicates of ~200 mg were placed into 20 mL scintillation vials for counts of incorporated  $^{14}\text{C}$ . The powdered sample was homogenized in 1 mL of distilled water and left overnight at room temperature. The next day, 1 mL of tissue solubilizer was added into each vial, which were then incubated in a water bath at 95 °C for 5 h before 10 mL "HiSafe"-III liquid scintillation cocktail was added. The homogenate solution was thoroughly mixed, left overnight and counted using a liquid scintillation counter (LSC) to determine  $^{14}\text{C}$  incorporation. Resulting counts per minute were converted to decays per minute using a quench curve and the external standard technique with correction for background radioactivity.

For the analysis of  $^{14}\text{C}$  in feces, 1 mL of 5% HCL was added into each vial containing feces and left overnight to breakdown any possible undigested algal cells. Ten milliliters "HiSafe"-III liquid scintillation cocktail was added into each vial and  $^{14}\text{C}$  was counted using LSC as above. The total  $^{14}\text{C}$  activity was summarized from all collections of feces during the 3 d pulse-chase feeding period.

#### Statistical Analysis

Assimilation efficiency (AE) was defined as the proportion of ingested  $^{14}\text{C}$  retained after completion of digestion and gut evacuation of  $^{14}\text{C}$  within 3 d, as

$$AE = \frac{{}^{14}\text{C}_{\text{flesh}}}{({}^{14}\text{C}_{\text{flesh}} + {}^{14}\text{C}_{\text{feces}})}$$

where  ${}^{14}\text{C}_{\text{flesh}}$  is  $^{14}\text{C}$  incorporated in the flesh and  ${}^{14}\text{C}_{\text{feces}}$  is  $^{14}\text{C}$  egested in the feces during the 72 h pulse-chase feeding period. This method is based on the assumption that respired and excreted  $^{14}\text{C}$  are negligible (e.g., Wang & Fisher 1996, Charles & Newell 1997). For example, Charles & Newell (1997) showed that respired  $^{14}\text{C}$  contributed as little as 0.4% to the total ingested  $^{14}\text{C}$  in the mussel *Geukensia demissa*.

Following Wang & Fisher (1996), gut passage time (GPT) is defined as the time at which 90% of the accumulated  $^{14}\text{C}$  in the feces is recovered, assuming 100% recovery at 3 d pulse-chase feeding period.

Multiple comparison tests on data were used to detect significant differences of assimilation efficiency between food algal types (95% confidence level). Linear regression model was used to analyze correlation between AE and GPT. All analyses were done using S-PLUS. (Insightful Corp.)

## RESULTS

#### Defecation and Gut Passage Time

No pseudofeces were detected during the experiments indicating that the concentration of total particulate matter was below the threshold for pseudofeces production. The defecation of nine-tested phytoplankton species in mussels after radioactive pulse feeding is shown in Figure 1. In general, the unassimilated portion of ingested radioactive phytoplankton showed a rapid loss through feces within the first few hours. However, the defecation rate varied considerably among phytoplankton species, being the fastest on a diet of *Alexandrium minutum*, *Chaetoceros calcitrans* and *Skeletonema costatum*, whereas *Thalassiosira* sp. and *Gymnodinium catenatum* were egested with slow rates. This defecation pattern can be shown more clearly using gut passage time (GPT) because this showed a large variation between food types, ranging

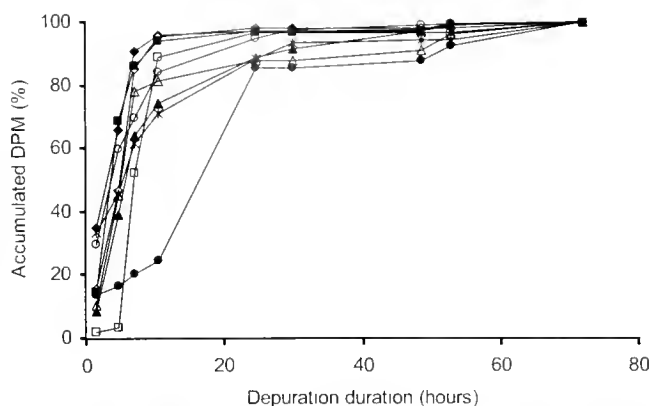


Figure 1. Time-series accumulated percentage of DPM in the feces after a pulse ingestion of radiolabeled phytoplankton by the greenshell mussel *Perna canaliculus*. Symbols:  $\blacklozenge$  = *Chaetoceros calcitrans*,  $\diamond$  = *Skeletonema costatum*,  $\blacktriangle$  = *Thalassiosira* sp.,  $\triangle$  = *Akashiwo sanguineum*,  $\blacksquare$  = *Alexandrium minutum*,  $\bullet$  = *Gymnodinium catenatum*,  $\square$  = *Eutreptiella* sp.,  $\circ$  = *Isochrysis galbana*, \* = *Pyramimonas* sp. Confidence intervals have been omitted from the graphs to improve clarity.

from 6.9–50.9 h (Fig. 2). Despite variation in GPT, species-specific differences were not significant ( $P > 0.51$ ). No significant differences in GPT were detected when the phytoplankton species were grouped into diatom, flagellate and dinoflagellate taxa ( $P > 0.71$ ).

#### Assimilation Efficiency

Assimilation efficiencies of the nine-phytoplankton species are shown in Figure 3. The greenshell mussel assimilated phytoplankton with overall high rates, but average assimilation efficiency (AE) varied considerably with phytoplankton species, ranging from 55.6% to 87.7%. Overall, AEs of dinoflagellates were highest, whereas AEs of diatoms were lowest. The average efficiency with which the mussel assimilated the dinoflagellates ( $84.5 \pm 6.3\%$ ) was significantly higher than that of diatoms ( $P < 0.01$ ) ( $61.7 \pm 11.0\%$ ). Similarly, mussels assimilated flagellates with significantly higher efficiency than diatoms ( $P < 0.01$ ). However, the difference of AEs between flagellates ( $77.9 \pm 6.7\%$ ) and dinoflagellates was not significant ( $P > 0.08$ ). There were no

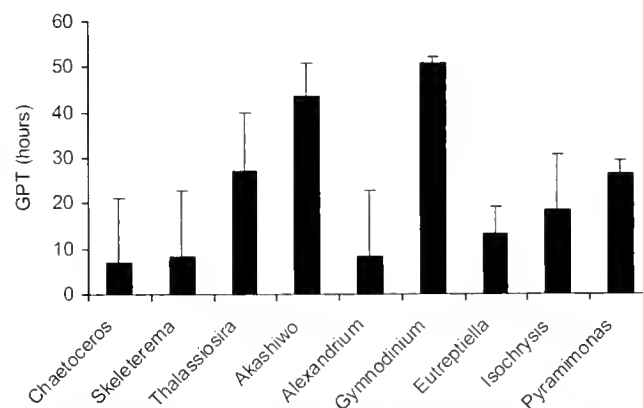


Figure 2. Variation of gut passage time (GPT) of the greenshell mussel *Perna canaliculus* fed with nine phytoplankton species. Error bars are  $\pm 1$  SE.

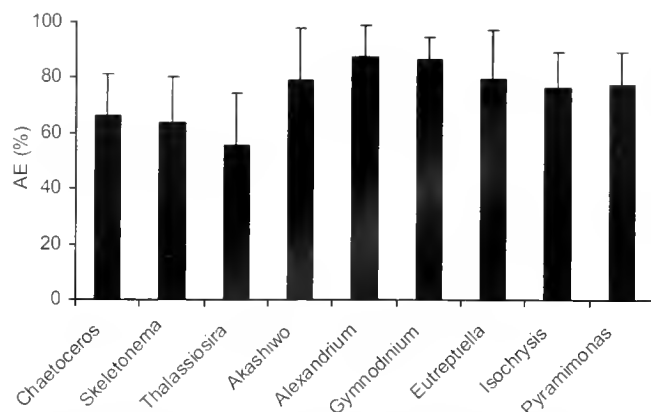


Figure 3. Assimilation efficiency of nine phytoplankton species by the greenshell mussel *Perna canaliculus*. Error bars are  $\pm 1$  SE.

significant differences of AE between species within diatom ( $P > 0.17$ ), flagellate ( $P > 0.56$ ) and dinoflagellate ( $P > 0.41$ ).

Correlation analysis showed that AE of diatoms was negatively correlated to GPT ( $R^2 = -0.97$ ,  $P > 0.11$ ), but AEs of dinoflagellates and flagellates were positively correlated to GPT ( $R^2 = 0.1$ ,  $P > 0.86$ ). If *Alexandrium minutum* is excluded, this trend is more robust ( $R^2 = 0.49$ ,  $P > 0.18$ ).

### DISCUSSION

The pulse-chase feeding technique was used in this study to specifically address the influence of phytoplankton species on assimilation efficiency in the greenshell mussel *Perna canaliculus*. We chose to use nine phytoplankton species to represent diatoms, dinoflagellates and flagellates that are found in the mussel farming areas in New Zealand. The results have demonstrated that AE varied between phytoplankton species, but AEs of dinoflagellates and flagellates was generally higher than those of diatoms.

The GPT in our study varied between 7 and 51 h, which was independent of algal species. Control of gut retention time has been demonstrated in *Mytilus edulis* and *Cerastoderma edule* and a strong positive relationship between absorption efficiency and gut retention time has been recorded in *M. edulis* (e.g., Hawkins et al. 1990, Wang & Fisher 1996, Chong & Wang 2000). Longer retention of ingested food within the digestive tract allows for more efficient digestion and assimilation (Willows 1992), and thus maximizes nutrient gain. Although this theory is supported by our experimental data that AEs of dinoflagellate and flagellate diets for which AE was positively correlated to GPT, AE of diatoms was negatively correlated to GPT. These results suggest that AE may not always increase with increasing GPT. Similar results have also been observed from two marine bivalves in which AE of pooled diatoms and flagellates was not correlated to GPT (Li et al. 2001). The assimilation efficiency of bivalves reflects complex physiological processes within an organism and can be influenced by many physiological factors including enzymatic digestive activity, gut residence time, gut capacity and digestive synchrony (e.g., Bayne & Newell 1983, Hawkins et al. 1990, Wang & Fisher 1996, Charles & Newell 1997). The adaptive strategies of bivalves in digestive processes (Bricelj et al. 1984) may help explain the differing results. A mussel would have little benefit in withholding relatively indigestible diatoms longer in the gut for more efficient digestion. The difference of diatom-AEs may have resulted from

the variability in digestibility between the species. Relatively less digestible diatom species may be egested more rapidly. Conversely, the longer GPT of relatively digestible dinoflagellates and flagellates would have resulted in more efficiency.

Our observations demonstrated that mussels assimilated carbon from diatoms at a lower efficiency than they did from dinoflagellates and flagellates. This is probably typical for many bivalves, because the cell walls of diatoms are rigid and resistant to enzymatic digestion and physical breakdown, and diatoms also contains significantly higher quantities of inorganic matter. AEs measured in our experiments are comparable with previous studies in other bivalves. For example, Shumway et al. (1985) found that bivalve molluscs preferentially assimilated a cryptomonad flagellate over a diatom. Variations in assimilation efficiency with different algal species have been observed in other marine bivalves (e.g., Romberger & Epifanio 1981, Wang & Fisher 1996). The assimilation of some algal species can be extremely low, which would result in low energy available for growth. For example, oysters *Crassostrea virginica* could hardly digest *Platymonas succica* (Romberger & Epifanio 1981). Results from our experiments indicated that the greenshell mussel digested algal species with a relatively overall high AE (55.6% to 87.7%) compared with the mussel *M. edulis* (7% to 86%, Wang & Fisher 1996), the oyster *C. virginica* (6% to 74%, Romberger & Epifanio 1981).

Although assimilation of the greenshell mussel have been investigated, direct comparison of AE is difficult because previous studies used either different algal species (Hawkins et al. 1999) or natural seawater (Gardner 2000) or combination of single algal species and natural seawater (Hawkins et al. 1999). To our knowledge, only one study used the same algal species (*Isochrysis*) as the present study (Marsden & Weatherhead 1999). Our measured AE (76.3%) is lower than theirs (84%). Differences in experimental designs may explain the differing results. The previous study used intertidal mussels starved for 3–12 d before experimentation. The physiological behavior may have either differed from farmed mussels or have been affected by starvation or both. Moreover, their study used the Conover technique. This technique relies on measuring gravimetric changes in the ratio of organic material to ash between food and feces, which is susceptible to weighing errors (Navarro & Thompson 1994). Therefore, it is not as sensitive as the radiotracer technique that we used in this study. Because the radiotracer technique does not require a large amount of material, it is particularly accurate when food concentration is low and consists of a high proportion of algal particles.

Selective feeding on particles of different sizes has been reported in filter feeders (Newell & Jordan 1989, Barrillé et al. 1993, Wang & Fisher 1996), but this selective behavior was only demonstrated in marine bivalves when fed with small particles. For example, experimental results by Barrillé et al. (1993) showed no change in retention efficiency of oyster (*Crassostrea gigas*) for particles larger than 3–4  $\mu\text{m}$ . Wang & Fisher (1996) demonstrated that assimilation efficiency of *M. edulis* was not directly related to the particle size of different algae ranging from 2–40  $\mu\text{m}$ , which is in agreement with other studies (e.g., Newell & Jordan 1983). Similarly, the size of the algal cells in our study ranged from 4–38  $\mu\text{m}$  (data not showed) and was not significantly related to assimilation efficiency ( $P > 0.68$ ). Therefore, algal sizes in this range may not be important during the digestive period and thus would not significantly affect assimilation efficiency.

We did not account for respiratory loss of  $^{14}\text{C}$  in the present

study, which may have resulted in slight underestimates of AEs. However, the respiratory loss of  $^{14}\text{C}$  may be negligible according to similar experiments on other mussels (e.g., Kreeger 1993, Wang & Fisher 1996) and only 0.4% of ingested food went to  $\text{CO}_2$  (Charles & Newell 1997). Although the contribution of respired  $^{14}\text{C}$  to AEs might be small, it is recommended that this term be included in the future measurements.

Studies on the food available to bivalve feeders in coastal waters reveal marked temporal variations in particulate matter concentration, composition and nutritional value (Bayne et al. 1993, Galois et al. 1996). Variability on a scale of days to weeks can result from episodic algal blooms and shifts in species composition. In the extensive mussel farming area of Marlborough Sounds, New Zealand, a high-frequency fluctuation of phytoplankton species composition has been observed (Mackenzie et al. 1986). For example, in early spring and summer of 1982 to 1983, microflagellates dominated the phytoplankton community (over 50%), whereas diatom species made up of less than 30% of the total biomass. In the autumn however, the diatoms dominated (>50%) (Mackenzie et al. 1986). An understanding of the influence of food type on assimilation efficiency is fundamental to validating models that predict not only greenshell mussel energetics and growth but also the capacity of the environment to support phytoplankton grazing by farmed mussels. Accurate models are essential for effective long-term farm management. There is a lack of comprehensive knowledge on assimilation efficiency and it is often assumed to be a constant or a functional response of ingested organic matter in many energetic modeling studies on farmed bivalves (Ren & Ross 2001, Ren & Ross 2005). An obvious and important aspect of the incorporation of AE into predictive models is obtaining values that are representative of the natural diet. Mussels have the ability to selectively ingest organic or high nutrient particles (e.g., Bayne & Hawkins 1990). If preferential assimilation of phytoplankton species has not been accounted for, then predictive models based on only one component of the diet such as organic content, may potentially under- or overestimate true energetics of the mussel. The present study has clearly demonstrated a

considerable difference of physiological responses of the green-shell mussel to algal types.

It should be noted that AE might vary not only with phytoplankton species but also with different life stages of bivalves. For example, the diatom *Skeletonema costatum* is an excellent food for oyster juveniles (Walne 1970) but poor for larvae of the same species (*Ostrea edulis*) that favor flagellates (Enright et al. 1986, Ferreiro et al. 1990). Assimilation efficiencies of the phytoplankton species tested in our study are applicable to adult mussels. Further experiments are needed to investigate how juvenile and larval stages of the mussel respond to changes of food types.

In conclusion, the present study shows that assimilation efficiencies of adult *P. canaliculus* depend on the phytoplankton genera and/or species. This variability appeared to be related to differing digestibility of phytoplankton species caused by rigid cell walls of diatoms being more difficult for enzymatic digestion than dinoflagellates and flagellates. This finding would provide important information to the understanding of the physiological response of the mussel to the type of phytoplankton species. However, a diet of a single algal species may evoke a feeding behavior that differs from diets of multialgal species or natural seawater (e.g., Romberger & Epifanio 1981, Navarro et al. 1992, Hawkins et al. 1996). Before the results are incorporated into energetic and carrying capacity models, further experimental work should be conducted to investigate the mussel responses to mixed diets of multialgal species in field conditions.

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## AGEING AND METABOLISM OF *MYTILUS EDULIS*: POPULATIONS FROM VARIOUS CLIMATE REGIMES

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**ABSTRACT** The aim of this study is to elucidate the impact of age and temperature on metabolic key parameters (respiration rate and mitochondrial marker enzymes) controlling maximal size and longevity in the blue mussel *Mytilus edulis* L. from geographically separated populations of Northern European seas. Mussels from the Barents Sea attain similar maximal sizes as animals from the warmer North Sea. Young animals (<5 y) grow faster in the North Sea, however, inshore Barents Sea mussels grow rapidly even at advanced age. The slowest growth rates were found in a population exposed to strong wave action in the Barents Sea. Respiration rates followed the same age dependent pattern in mussels from all studied populations with low respiration rates in the youngest specimens followed by a rapid increase and then a gradual decline to nearly constant values in the oldest animals. When compared at a common temperature, metabolic rates only of young blue mussels (<7–8 y) are cold compensated with maximal respiration in the most Northern populations. Cold compensated metabolic activities in mussels from Northern populations may support similar patterns of maximal body size and longevity as in the more temperate Southern population.

**KEY WORDS:** ageing, Barents Sea, citrate synthase, cytochrome C oxidase, growth, environmental temperature, *Mytilus edulis*, North Sea, respiration rate, White Sea

### INTRODUCTION

Most eurythermal marine invertebrates of the Northern hemisphere have wide latitudinal distribution and physiological differences between spatially separated populations of one and the same species can be pronounced. In times of rapidly changing regional climate regimes it is a prime concern to study the quality of physiological adaptations, especially in key species like the blue mussel *Mytilus edulis*. Low ambient temperatures slow down metabolic activity at high latitudes, and there is by now ample evidence that thermal lowering metabolic rates extends life span in ectothermal models like *Caenorhabditis elegans* (Van Voorhies & Ward 1999) or *Drosophila melanogaster* (Luckinbill 1998). However, in aquatic ectotherms observations are controversial. Some eurythermal species are distributed over wide latitudinal gradients, and, at least in northern populations, the animals tend to attain bigger body sizes and longer life spans than their southern counterparts (MacDonald & Thompson 1988, Gianniny & Geary 1992, Duchesne & Magnan 1997). In others, no tendency or the opposite trend is observed (Selin et al. 1991, Garvey & Marschall 2003). Although a growing number of publications analyze the ageing process and the influence of age on metabolism in ectotherms with infinite growth (Zolotarev & Ryabushko 1977, Fidhiany & Winckler 1998, Zielinski & Pörtner 2000, Sukhotin et al. 2002, Philipp et al. 2005, 2006), virtually nothing is known about the variation of ageing parameters within a species with a latitudinal distribution.

Latitudinal variations of metabolic rate in aquatic ectotherms were extensively studied and shaped into the hypothesis of metabolic cold adaptation, which suggests a significant elevation of metabolic rate in cold adapted species. This hypothesis has recently been challenged for Antarctic stenotherms (Clarke & Johnston 1999, Peck & Conway 2000), but it has been re-emphasized in comparative studies of temperate and subpolar,

Northern hemisphere eurytherms (Pörtner 2002a, Pörtner 2004). Notably, eurythermal cold-water animals are characterized by elevated standard metabolic rates in comparison with temperate and tropical confamilial species. The present study addresses the consequences of these patterns for the lifespans of marine bivalves by investigating metabolic changes in the course of ageing in geographically separated populations of the blue mussel *Mytilus edulis*. *M. edulis* is a eurythermal species inhabiting European coasts from the French Atlantic coast in the South to the eastern parts of the Barents Sea and the White Sea in the North. We compared growth, metabolic rates and mitochondrial key enzymes of blue mussels from the relatively warm temperate North Sea (Helgoland), the cold subArctic White Sea, where seasonal temperature variations can span up to 15°C, as well as from the cold, but thermally more stable Barents Sea (the seasonal difference in temperature is about 6°C).

### MATERIAL AND METHODS

#### Animals

Field sampling of blue mussels *Mytilus edulis* L. was carried out in 3 main areas: (1) Barents Sea (Kola Peninsula, Yarnyshnaja Bay 69°06'N, 36°04'E), (2) White Sea (Chupa Inlet of Kandalaksha Bay, 66°20'N, 33°40'E) and (3) North Sea (at Helgoland Island, 54°11'N, 7°53'E) (Fig. 1a). Salinity of surface waters range from about 24 ppt in the White Sea to 28–32 ppt in the Barents and the North seas. The three selected sampling locations form a cline of temperature conditions. The warmest is the North Sea, whereas the White and the Barents Seas are characterized by far lower winter and summer temperatures, respectively (Fig. 1b). At each site dense or dispersed settlements of *Mytilus edulis* are found in intertidal and upper subtidal zones. Sampling was performed from intertidal dense mussel beds at about 0.7–1 m above 0 tidal level in each of the three sampling areas. The mussel bed in Yarnyshnaja Bay (Barents Sea, BSi) is situated in the innermost part of a narrow bay, which penetrates about 6 km into the Kola Peninsula shoreline and is thus protected from wave action. In the White Sea sampling

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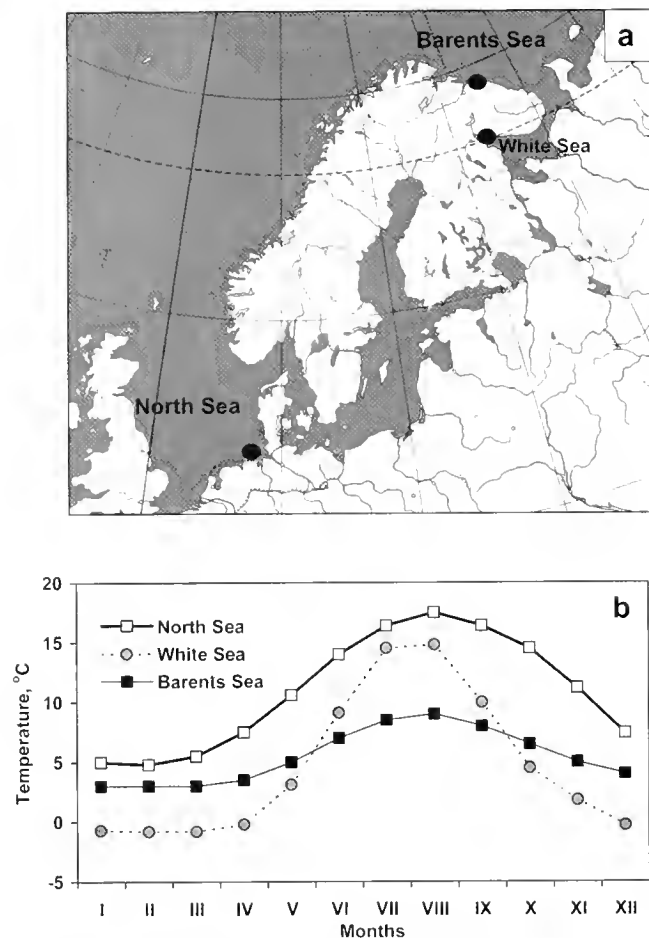


Figure 1. Map of study area and sampling sites of mussels (a) and monthly average temperature in the North Sea (<http://bah.wdc-mare.org>), Barents Sea (Matishov et al. 1998) and White Sea (Babkov, 1982).

area (WS) *Mytilus edulis* forms a dense multilayer population on the southern shore of a small island, which is also sheltered from direct wave action, whereas exposed to strong tidal currents. In the North Sea (NS) mussels were collected from the rocky intertidal on the NW coast of Helgoland Island. This site is exposed to wave action at N to NE wind directions.

Mussels were collected in October (BSi and WS) and November (NS) 2002. Sea water temperature was +5.5°C and +9°C, respectively. Furthermore, mussels were sampled from an intertidal population situated in the outer part of Yarnyshnaja Bay of the Barents Sea (BSO). This area is exposed to strong wave action. In addition, a small sample of mussels from the most Eastern part of the Barents Sea was kindly collected by Dr. P.P. Strelkov. These mussels were sampled from subtidal population in 6-m depth near the Vajgach Island (Barents Sea, Novaya Zemlya Archipelago, 69°44'N, 59°30'E) in August 2002 at a water temperature of +3°C.

Molluscs from all sites were transported alive to the Alfred-Wegener Institute (Bremerhaven, Germany) and maintained in aquaria at 30 ppm salinity and +5.7°C temperature. Age was determined for all mussels by counting the rings of winter growth delays on the outer shell surface. This method has previously been verified in studies carried out in the White Sea by comparing the number of internal rings and seasonal growth of individually marked mussels (Chemodanov & Maximovich 1983, Sirenko &

Saranchova 1985). Respiration measurements were performed in January 2003.

#### Growth

Growth rings of mussels from all four populations were measured from the umbo to the most distant edge of the shell to the nearest 0.1 mm for reconstruction of the growth history of mussels. Linear growth of mussels was approximated using the von Bertalanffy model

$$L_t = L_{\infty} \cdot (1 - \exp^{-k \cdot (t - t_0)}),$$

in which  $L_t$  is length (mm) at the age  $t$  (years),  $L_{\infty}$ ,  $k$  and  $t_0$  are constants.

#### Experimental Procedure

Mussels from all populations were sorted by age with considerable variability in animal size within each age class. The BSi group included age classes from 3–15 y (12 and 14 y age classes were missing) ranging in size from 0.187–4.740 (mean = 1.03) g wet tissue weight. The BSo group was represented by 4–8-y-old mussels (0.095–0.479, mean = 0.29 g wet tissue weight). Mussels from the White Sea (WS) were 1–10 y old (0.046–2.40, mean = 0.72 g wet tissue wt) whereas the North Sea sample (NS) comprised animals from <1 to 11 y of age (0.206–4.036, mean = 1.58 g wet tissue wt). Vajgach sample (Vajgach) was represented by only 8 specimens 5–11-y-old (0.249–1.374, mean 0.75 g wet tissue wt). Mussels from this sample were used only for respiration rate measurements.

Oxygen consumption was measured at +5°C in closed 150–200 mL respirometers, containing 1–6 mussels. Oxygen concentration was monitored using Clark type oxygen electrodes (Eschweiler, Kiel, Germany). After the measurements mussels were cut open, gills were dissected, blotted dry on tissue paper, weighed to the nearest 0.1 mg and deep frozen in liquid nitrogen for further analyses of enzyme activities. The residual tissues were also weighed. Respiration rate was expressed in  $\mu\text{mol O}_2 \text{ g}^{-1} \text{ tissue wet wt h}^{-1}$ .

#### Analyses

Citrate synthase (CS, EC 2.3.3.1) activity was measured spectrophotometrically at 412 nm using an enzymatic test (Sidell et al. 1987). Powdered tissue (about 100 mg) was homogenized in 75 mM Tris+1mM EDTA buffer (pH 7.6) at a 1:9 w/v ratio. After centrifugation (10 min, 9,500 g, at 0°C) CS activity in the supernatant was measured in 100 mM Tris-HCl buffer (pH 8.0) containing 20mM acetyl-CoA, 20 mM oxaloacetate and 5 mM DTNB. Absorbance was monitored at 412 nm using a spectrophotometer (LKB Pharmacia) with cuvettes thermostatted at +25°C.

Cytochrome C oxidase (COX, EC 1.9.3.1) activity in mussel gill tissue was determined according to Hardewig et al. (1999). Powdered tissue (1:9 w/v) was homogenized in 20 mM Tris-HCl +1mM EDTA + 0.1% Tween 20 buffer (pH 7.4). Enzyme activity was measured in the assay buffer (20 mM Tris-HCl, pH 8.0 + 0.5% Tween 20 + 50  $\mu\text{M}$  reduced cytochrome c) at +25°C. The decrease of absorbance was determined spectrophotometrically (LKB Pharmacia) at 550 nm. Cytochrome c, dissolved in 20 mM Tris-HCl, pH 8.0 was reduced by sodium dithionite, which was removed by gel filtration in a Sephadex G-25 column. Enzyme activities were expressed in  $\text{U g}^{-1} \text{ tissue wet wt}$ .

#### Calculations and Statistics

One-way ANOVA was used to compare densities, biomasses, respiration rates and enzyme activities between populations. Post-



hoc comparisons were made by Tukey HSD Test for unequal N. Age effects on respiration rates and enzyme activities were tested by Kruskal-Wallis nonparametric test. Age distributions in the studied populations were compared by the Chi-square test. The F-test was used for comparisons of linear growth equations in different samples.

## RESULTS

### Growth

Mussels from NS and WS populations grew rapidly during the first 4–5 y but growth tended to level off hereafter (Fig. 2). Molluscs from the Barents Sea displayed similar growth strategies in both populations until the 5th year, when animals from the open shore (BSO) slowed their growth, whereas mussels from the sheltered part of the bay (BSi) continued intensive growth until the age of about 10 y, whereafter a decrease in growth rate was observed. In terms of absolute shell length increment, mussels from the North Sea had the fastest growth of all populations in the first 2–3 y after settlement, attaining over 30-mm body length during young age. Hereafter their growth rate diminished to that of mussels from the other sites. The most rapid growth at advanced age (over 5-y-olds, beyond 20-mm body length) was found in the Barents Sea population from the protected site in Yarnyshnaja Bay (BSi). Pairwise comparisons showed that growth curves of mussels from the White Sea (WS) and from the open part of the Yarnyshnaja Bay (BSO, Fig. 2) did not differ statistically ( $P = 0.246$ , F-test). Both demonstrated an abrupt decrease of growth rate after reaching 5 y of age. Growth rates of BSi mussels differed significantly from both BSO and WS specimens at  $P = 0.0012$  and  $P < 0.001$ , respectively, whereas NS animals differed from all others at  $P < 0.001$  (F-test).

### Respiration Rate

The effect of body size on respiration was approximated using 5-y-old individuals from all 3 stations, because this age class was in the middle of the age range and also one of the most abundant (except for the juveniles) in the studied populations (unpublished data). Respiration rate ( $RR$ ,  $\mu\text{mol O}_2 \text{ g}^{-1} \text{ wet wt h}^{-1}$ ) of 5-y-old *M. edulis* decreased as a function of tissue wet weight ( $W$ , g)

$$RR = 3.8 W^{-0.356} \quad (r = -0.838, p = 0.001, N = 15).$$

The obtained power coefficient ( $-0.356$ ) was then used for a weight correction of the respiration data to a mean wet tissue weight ( $= 1.00 \text{ g}$ ) for comparisons between mussels of different ages and populations. The correction was as follows  $R_{\text{corrected}} = R_{\text{observed}} \times (1/W)^{0.356}$ .

Although the Vajgach population was subtidal and therefore differed from the other populations, it was included in the inter-population comparisons of mussels' respiration as the most distant outpost with the lowest ambient temperatures. The factor "population" had a significant ( $P < 0.001$ , ANOVA) effect on weight-corrected respiration rates. Figure 3a presents respiration rates of mussels from 5 populations as means of all ages. Respiration increased in the following rank order: NS < WS = BSi = BSO < Vajgach. Compared at a common experimental temperature of 5 °C, NS mussels were characterized by significantly ( $P < 0.01$ , Tukey HSD) lower respiration rates than their more northern counterparts, whereas Vajgach animals exceeded NS, WS and BSi ones by respiration at  $P < 0.05$ . The factor "population" affected mussel respiration in the separate age classes: 2, 4, 5, 6 and 7 y ( $P < 0.05$ , Kruskal-Wallis test), whereas at ages 1, 3 and older than 7 y the difference between populations was below significance (Fig. 3b).

Because age classes in Vajgach sample were represented by too

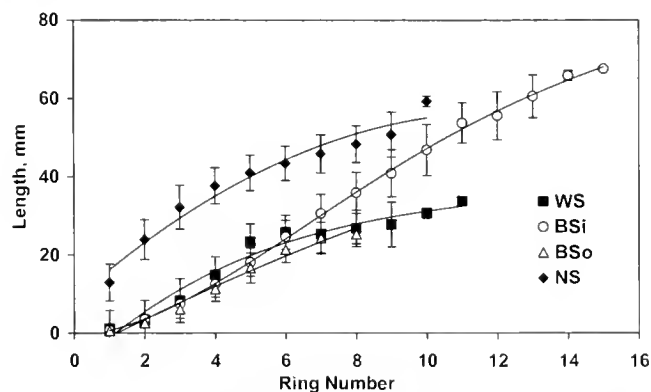


Figure 2. Von Bertalanffy growth curves in mussel populations. NS, North Sea;  $L_{\infty} = 62.8 \cdot (1 - \exp^{-0.197(t+0.34)})$ ,  $n = 215$ . BSi, Barents Sea; innermost (sheltered) part of the bay;  $L_{\infty} = 412.0 \cdot (1 - \exp^{-0.014(t-1.380)})$ ,  $n = 194$ . BSO, Barents Sea; outer (open) part of the bay;  $L_{\infty} = 126.2 \cdot (1 - \exp^{-0.035(t-1.206)})$ ,  $n = 30$ . WS, White Sea;  $L_{\infty} = 39.3 \cdot (1 - \exp^{-0.181(t-1.160)})$ ,  $n = 238$ . Data are mean  $\pm$  SE.

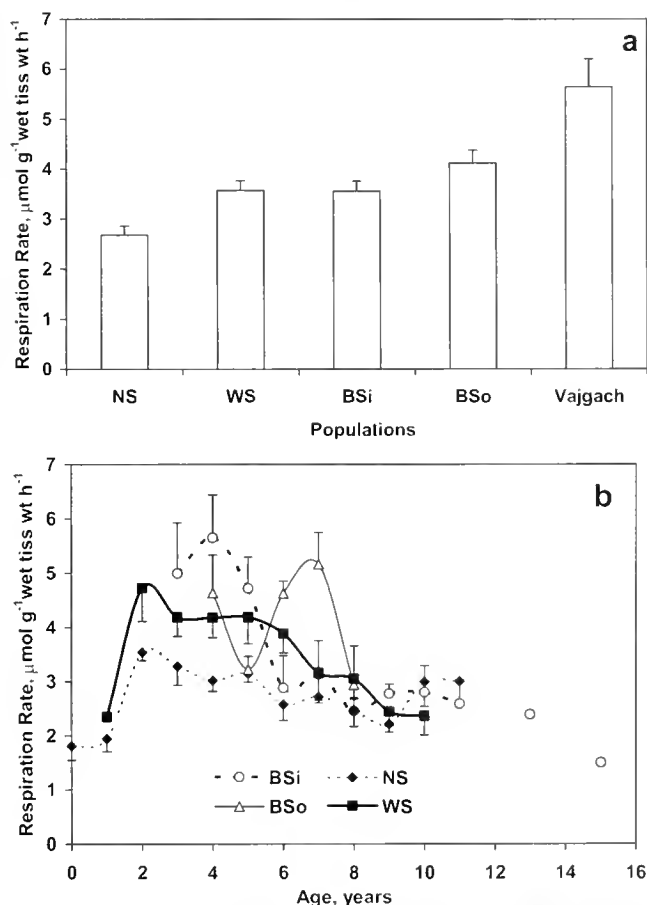


Figure 3. Respiration rates of mussels from studied populations—all age classes combined together (a) and in different age classes (b). NS, North Sea; BSi, Barents Sea; innermost (sheltered) part of the bay. BSO, Barents Sea; outer (open) part of the bay. WS, White Sea; Data are mean  $\pm$  SE, evaluated at 5 °C.

few specimens this population was not included in the analysis of age influence on respiration. Age effects on mussel respiration rates were significant at  $P = 0.026$ ,  $P = 0.047$ ,  $P = 0.004$  and  $P = 0.010$  (Kruskal-Wallis test) for BSi, BSo, NS and WS populations, respectively (Fig. 3b). The shape of the curves describing the age dependence of respiration rates in mussels was similar for NS and WS molluscs. In these groups respiration rates were low at young age (0–1-y-old) followed by a significant increase in 2-y-old specimens. Older mussels were characterized by a gradual decrease of respiration with age, with constant rates in age classes 3–5 in both WS and NS molluscs. The oldest (10–11-y-old) specimens from the NS displayed insignificantly higher respiration rates than 6–9-y-old animals from that same site. However, the sample size was too small for meaningful statistical analyses.

The respiration rate of the youngest mussels in the BSi group (3-y-old) was somewhat, but nonsignificantly, lower than that of 4-y-old specimens, which displayed the highest respiration rates in this population. A rapid 2-fold decrease in respiration rate was recorded between age classes 4 and 6 followed by a gradual decline to the lowest values seen in 15-y-old specimens.

The age range of mussels from the BSo population was much narrower than that of the other populations and included only age classes 4–8 y. Animals older than 8 y were never found on the BSo site. On the other hand, young specimens (0–3-y-old) were too small to be included in the experiments (mean shell length of 3-y-olds from BSo was 6.1 mm). The respiration rate of BSo mussels was similar in all age classes

#### Enzyme Activities

CS activity in gill tissue of *M. edulis* varied from 2.15–8.24  $\text{U g}^{-1}$  wet tissue wt, whereas COX activity was in the range 0.44–1.65  $\text{U g}^{-1}$  wet tissue wt. The activity of both enzymes appeared positively correlated (Spearman  $R = 0.517$ ,  $n = 125$ ,  $P < 0.001$ ) (Fig. 4). Size effects on the activity of citrate synthase and cytochrome C oxidase were tested using regression (enzyme activity vs. body mass) analyses in separate age groups of different populations. No significant trend was found for either CS or COX.

Age of the mussels had no influence on either CS or COX activities (Kruskal-Wallis test) (Fig. 5a). The factor "population" significantly affected the activity of both enzymes (ANOVA,  $P < 0.01$  for CS and  $P < 0.001$  for COX) resulting in a decrease in activity in the order  $\text{NS} > \text{WS} > \text{BSi} \geq \text{BSo}$  (Fig. 5b). NS mussels differed from both Barents Sea populations ( $P < 0.05$ , Tukey HSD

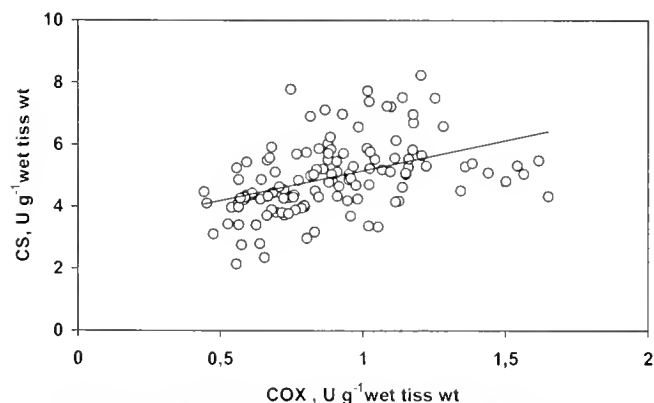


Figure 4. Relationship between cytochrome C oxidase and citrate synthase activities in mussels analyzed at 25 °C.

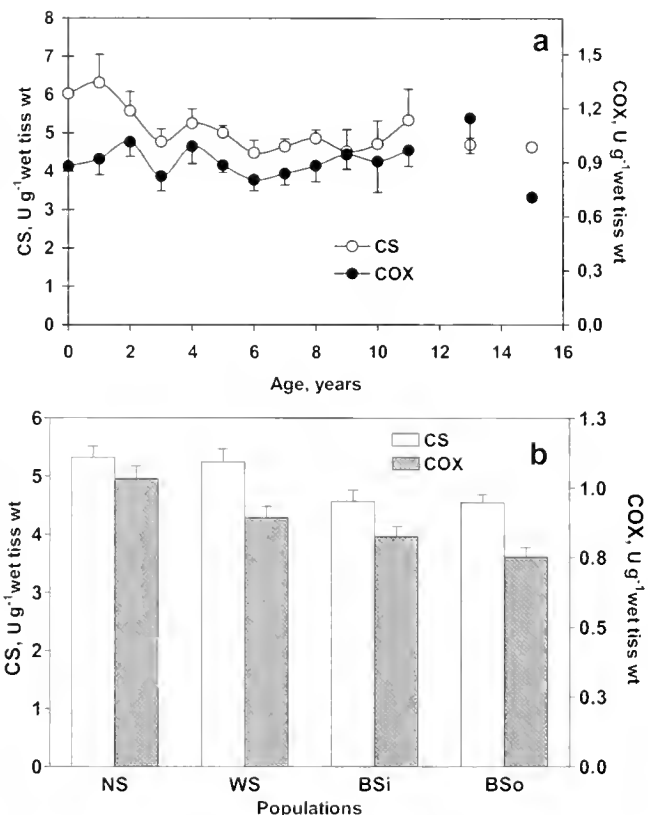


Figure 5. The effect of age (a) and population (b) on cytochrome C oxidase and citrate synthase activities in mussels (enzyme activities analyzed at 25 °C).

test) with respect to COX and from BSi ( $P < 0.05$ , Tukey HSD test) with respect to CS activities.

#### DISCUSSION

Growth patterns and maximal size observed in the NS and WS populations are typical for mussels from intertidal habitats of the respective areas (see for comparison, for the North Sea: Craeymeersch et al. 1986; Munch-Petersen & Kristensen 2001, for the White Sea: Savilov 1953, Maximovich 1989). On the exposed rocky shore (BSo) mussels colonize crevices in the rocks, where they are protected from strong wave action. Maximal size of mussels in this habitat is obviously limited by the sheltered space available, because animals sticking out from crevices are mechanically removed by waves and winter ice. Strong hydrodynamic action on open shores provide better conditions for growth of mussels from different species than sheltered bays, however, in extreme cases growth on exposed habitats is inhibited and longevity is reduced (McQuaid & Lindsay 2000, Steffani & Branch 2003). Growth of mussels from the sheltered BSi population is similar to that of their conspecifics from the exposed BSo population until the animals reach about 20-mm shell size at 5–6 y. Older BSo animals grow significantly slower than BSi mussels and have only 2 y of further life expectancy. Thus, maximum size and longevity of mussels from the exposed intertidal of the Barents Sea are likely determined by wave action and firmly limited to a maximal size of 30–35 mm reached after 8 y.

In contrast, animals from BSi do not seem to be limited by any mechanical factor and reach maximal old age and maximal attain-

able size among all studied populations. Although before 3–4 y of age they grow slower than NS and WS mussels with smaller annual growth increments, they keep growing by about 5–6 mm per year until they are about 13-y-old and, eventually, outgrow and outlive mussels from all other populations of this study.

NS mussels exceeded all other populations in shell size within each age class. This difference is grounded in a significant distinction in body size reached by the end of their first growth season in all four populations. *Mytilus* spat in the NS had grown to a mean size of 12.9 mm by the beginning of winter, whereas newly settled mussels in the WS, BSi and BSo attained on average 1.1, 0.81 and 0.60 mm during their first growth season. This parameter (the size of the first ring) depends on two factors: growth rate and duration of the growth season. In the North Sea (at Helgoland, see Sprung, 1983) *Mytilus* settlement starts already in midMay resulting in a 4–5 mo long initial growth season for the spat in a relatively warm sea. In the White Sea and the Barents Sea mussels spat settles in August and even later, leaving not more than 1.5–2 mo for growth before seasonal cooling starts.

According to Bergmann's rule (originally elaborated for mammals and birds, Ray 1960) animals tend to increase their average body size with increasing latitude. Among Northern hemisphere aquatic ectotherms following this rule are fishes *Squalus mitsukurini* (Taniuchi et al. 1993), and the gastropod mollusc *Acanthina punctulata* (Gianniny & Geary 1992). Some weaker but significant trends to grow bigger at higher latitudes are recorded for the giant scallop *Placopecten magellanicus* (MacDonald & Thompson 1988) and the deep-sea gastropod *Troschelia berniciensis* (Olabarria & Thurston 2003). There are however numerous deviations from this rule (e.g., Gilbert 1973, Selin et al. 1991, Duchesne & Magnan 1997, Garvey & Marshall 2003). The largest specimens of mussels found in our samples from the NS and BSi populations were 60.0 and 71.4 mm length (4.32 and 4.74 g wet tissue weight), respectively. Although mussels from the northern population (BSi) in our study reach the biggest shell size, they attain only similar maximal soft body masses as NS mussels. Therefore, our data do not support Bergmann's rule as well as the expectations of slower growth and longer life of mussels from Arctic compared with temperate populations.

Age effects on respiration rates of mussels were studied in four different populations over a wide age range from 0–15 y. In all populations a similar pattern was observed, namely, low respiration rates in the youngest specimens followed by a rapid increase and then a gradual decline to nearly constant and similar values in the oldest specimens at each site. Initial low respiration values may relate to sexual immaturity of the young animals. It has been shown (Maximovich 1985) that *M. edulis* in the White and the Barents Seas attain sexual maturity only beyond 15–20 mm shell length (50–100 mg wet tissue weight). 2-y-old mussels from the WS population included in the analysis displayed 160–340-mg tissue wet weight, whereas 1-y-old mussels ranged between 40 and 60 mg. The fact that 2-y-olds were already mature, whereas 1-y-olds were immature can explain the difference in respiration rates between mussels of these two age classes. According to Sprung (1983), mussels from Helgoland become sexually mature after 1 or 2 y, which explains low respiration rates in 0–1-y-old specimens from the NS. The youngest mussels (3 and 4-y-old) studied in both Barents Sea populations were mature and displayed high respiration rates.

The age-related pattern of respiration rates is common among all four studied populations. Sexual maturation is accompanied by

an increase of weight specific respiration, which is followed by a more or less pronounced decrease of respiration at older age. It is interesting that despite the almost 2-fold difference in maximum lifespan observed in the studied populations (8 and 15 y in BSo and BSi, respectively), the age window of "metabolically most active" mussels is the same in all populations (from the age of sexual maturity to 6–7 y) (Fig. 3b).

A general decrease of weight specific metabolic rate in the course of ageing is reported for homeotherms and insects (see for review McCarter 1995), however, with few exceptions (O'Connor et al. 2002). Data on age-dependence of metabolic rate in infinitely growing species are controversial. Mere age effects on respiration rate (not linked to the influence of body size) were reported for bivalves *Crenomytilus grayanus* (Zolotarev & Ryabushko 1977), *Argopecten irradians irradians* (Bricelj et al. 1987), *Mytilus edulis* (Sukhotin & Pörtner 2001) and fish *Cichlasoma nigrofasciatum* (Fidhiany & Winckler 1998). However, in other studies this effect was poor or not seen at all (Pérez Camacho et al. 2000, Sukhotin et al. 2002, 2003). This study shows that age effects on metabolic rate of mussels are not straight forward (the older the animal, the lower the metabolic rate). There can be periods with a strong effect of age on respiration (e.g., an increase after maturation or a rapid decrease after 6–7 y of age) and also time intervals when metabolism is virtually independent of age (e.g., in the most metabolically active and also in the oldest specimens). Therefore, comparisons of selected age classes in latitudinal comparisons, seeking for an effect of environmental temperature on metabolic rates may lead to erroneous interpretations. Further, our data suggest that the age-related decline in respiration is more pronounced in cold-adapted subArctic animals (BS and WS) than in North Sea mussels. Moreover, in warm-acclimated (summer) animals (Sukhotin et al. 2002; 2003) the age effect may not appear as pronounced as in late autumn (this study) and winter.

Respiration rates of mussels from the investigated populations are indicative of metabolic cold compensation in *Mytilus edulis* from Northern latitudes. This compensation of respiratory capacity in cold adapted ectotherms from the Northern hemisphere is achieved by an increase of mitochondrial density or inner membrane surface area as well as mitochondrial capacity (Tschischka et al. 2000, Sommer & Pörtner 2002, 2004). Mussels from intermediate reproductive age classes from the more northern populations had higher specific respiration rates than those from the North Sea. By contrast, old and presumably also very young mussels had lower and uniform respiration rates in all populations so that population differences were abolished in the old animals (Fig. 3b). In other words, cold adaptation, specifically latitudinal differences in metabolic rates according to different temperatures manifests especially in the most metabolically active specimens, possibly because of active somatic growth and substantial reproductive effort, which are costly in terms of assimilated energy. When combined, both findings support the view that in the Northern *Mytilus* populations lifetime energy expenses peak early in life to support reproductive output in young animals, whereas metabolic activity declines rapidly thereafter. This may be an adaptation to the harsh environmental conditions and may also reflect an age-related decrease of energy demands for somatic growth and reproduction, until a baseline level of energy turnover is reached as required for maintenance that allows no further reduction in the old animal.

Weight specific activities of metabolic enzymes as citrate synthase and cytochrome C oxidase often vary between warm and cold adapted mollusks or between summer and winter specimens

in one and the same population. Interestingly, whole animal respiration in our study did not follow the same trend as the mitochondrial key enzymes in gills. Enzyme activities in gills were highest in the North Sea population (25°C assay temperature) but whole animal respiration at 5°C was lower at Helgoland than in WS and BS animals. Higher enzyme activities at low whole animal respiration rate would suggest higher mitochondrial densities or capacities in gills of the warmer North Sea animals despite low respiration rate measured at 5°C. This apparent contradiction might suggest that the metabolic requirements in gills vary independently from those of the whole animal. It appears conceivable that food demand and associated gill activity are high at the higher temperatures of the North Sea such that gill metabolic capacity is set high. Moreover, according to the temperature regime seen at Helgoland roads (Wiltshire & Manly 2004: 3°C to 20°C, mean  $T = 9^\circ\text{C}$ ) the temperature applied during respiration analyses is low for Helgoland indicating that metabolic rate was measured at the low end and enzyme capacities were analyzed beyond the high end of the thermal window. Future analyses should compare mussel metabolism and enzyme activities at the same and within the range of habitat temperatures.

Aerobic enzyme capacities in other invertebrates have been found to vary in line with the cost of cold eurythermy hypothesis (Pörtner et al. 2000, Pörtner 2004). Among scallops, COX activity ranged significantly higher in *Aequipecten opercularis* from the warmer Irish Sea than in cold adapted stenothermal *Adamussium colbecki* from the high Antarctic, whereas CS activities were similar in both species (Philipp et al. 2006). In contrast, cytochrome C oxidase (COX) activity was 10-times higher during summer in cold adapted eurythermal lugworms *Arenicola marina* from the White Sea at 6°C than in North Sea specimens measured at 11°C (Sommer & Pörtner 2002). Seasonal influences may modulate this picture. Sommer and Pörtner (2004) and Keller et al. (2004) found that mitochondrial respiratory capacity was significantly higher in prespawning and lower in postspawning summer animals than in

winter. Concomitantly, CS activities in North Sea lugworm tissue during summer versus winter were equal prespawning or lower postspawning. A seasonal comparison of the related horse mussels, *Modiolus modiolus*, (Mytilidae) from shallow subtidal environment (10-m depth) of New Hampshire, Isle of Shoals, United States (Lesser & Kruse 2004) found somewhat but significantly higher respiration in summer than winter animals, however, again with CS concentration per g tissue significantly increased in the winter. This may reflect reduced whole animal respiration rates caused by metabolic depression in winter, whereas enhanced CS levels and COX activities during winter (Lesser & Kruse 2004) are in line with elevated capacities of mitochondria in seasonal cold and may also meet additional metabolic requirements on top of supporting mitochondrial capacity (Pörtner 2002b).

In conclusion, the compensatory increase of metabolic rate in cold adapted Northern hemisphere mussels manifests especially in the most metabolically active individuals and is less apparent at advanced age. Our data suggest compensation for latitudinal cold during summer in blue mussel *Mytilus edulis* Northern populations. Cold compensation is mostly reflected in active whole animal respiration rates, in line with the cold eurythermy hypothesis (cf. Pörtner et al. 2000, Sommer & Pörtner 2004). According to our data, growth and longevity in blue mussels relate to temperature variability within the climatic range covered in this study as well as to local environmental factors. Similarity of maximal life spans of mussels from latitudinally separated populations can be explained by similar metabolic rates at ambient temperature caused by metabolic cold compensation in animals at high Northern latitudes.

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## VALVE-GAPE RESPONSE TIMES IN MUSSELS (*MYTILUS EDULIS*)—EFFECTS OF LABORATORY PRECEDING-FEEDING CONDITIONS AND *IN SITU* TIDALLY INDUCED VARIATION IN PHYTOPLANKTON BIOMASS

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**ABSTRACT** Many physiological processes in bivalves are influenced by the valve opening state and thus the valve opening-closing response times of the experimental animals. Controlled laboratory studies using underwater video camera recording of valve-gape responses of mussels (*Mytilus edulis*) to presence or absence of algal cells in the ambient water have revealed that valve opening and closing responses are strongly influenced by the preceding feeding conditions. The observations indicate that the period during which mussels may “learn” or “forget” to respond to the presence or absence of algal cells can last for weeks in the laboratory. Further, the critical algal concentration below which mussels close their valves has been identified to be about 700 *Rhodomonas* sp. cells mL<sup>-1</sup>, or 0.9 µg chl *a* L<sup>-1</sup>. Such knowledge is essential for making allowance for possible deviation from natural-response times in mussels kept in the laboratory previous to experimental physiological studies. *In situ* simultaneous registrations of water flow, chl *a* and valve-opening degree of mussels were made within a dense mussel bed in a shallow, tidally dominated inlet to a Danish fjord. Phases of strong tidal flow brought ample supply of phytoplankton to the mussel bed, and it was observed that concentrations higher than about 1 µg chl *a* L<sup>-1</sup> in the near-bed layer stimulated the mussels to keep their valves wide open, whereas tidal slack with slow flow resulting in near-bed concentrations below 1 µg chl *a* L<sup>-1</sup> caused by grazing by the mussels, after some time led to shell closure, or reduced valve gapes of the mussels. The valve opening time from opening degree 50% to 100% and the valve closing time from opening degree 100% to 50% in response to variation in near-bed chl *a* concentration were on average 59 ± 22 and 50 ± 19 min, respectively, which is considerably faster than observed in unfed mussels in the laboratory.

**KEY WORDS:** feeding activity, response to phytoplankton, valve gape, feeding activity, grazing impact

### INTRODUCTION

Many physiological processes in bivalves are strongly influenced by the valve opening state and thus the valve opening-closing response times of the experimental animals (Riisgård 2001b). Filter-feeding bivalves are able to sense the presence of suspended food particles in the ambient water, and this sensitivity is reflected in the gape of the valves (Jørgensen 1975). The behavioral valve-opening repertoire ranges from closed valves and retraction of mantle edges and siphons to fully open valves accompanied by extended mantle edges and siphons (Jørgensen et al. 1988). Under optimal conditions, bivalves filter the ambient water at a maximum rate and under sub optimal environmental conditions, including low or (very) high concentrations of phytoplankton, the valve gape is reduced and the mantle edges retracted (Riisgård & Randlov 1981, Jørgensen et al. 1988, Riisgård, 1991, 2001a, Clausen & Riisgård 1996, Newell et al. 1998, 2001, Dolmer 2000a, 2000b, Riisgård et al. 2003, van Duren et al. 2005, Lassen et al. 2006). Although mussels in nature may often experience phytoplankton concentrations below the threshold level, the valve gape opening-closing response to natural variations in phytoplankton biomass in nature has so far been unobserved.

In algal clearance experiments with blue mussels, *Mytilus edulis*, we have often over the years observed that when mussels are fed algal cells for some hours every day during a period of up to one week, as has been the case during many student laboratory exercises, both the opening and closing response apparently become faster than observed on mussels starved for some days—or sometimes weeks—previous to the first clearance experiments. These observations draw attention to the importance of the previous feeding history of the bivalves. More knowledge on the response times in mussels will improve our understanding of the role of mussels for the energy flow in highly dynamic systems. In such

systems, the opening-closing-response times to presence/absence of food may potentially limit ingestion and the grazing impact on phytoplankton. Awareness of response times is also important when interpreting (e.g., observed correlations between low food concentrations and clearance rates in mussels). On shorter time scales (minutes to hours) the mussel response times may cause hysteresis and mask the underlying functional response of clearance rate to food concentration.

In the present work we first in controlled laboratory experiments examined if mussels are able to lose and regain the ability to respond promptly to absence and presence of suspended algal cells in the ambient water. Such knowledge about “memory/learning/forgetting” in filter-feeding bivalves may be essential for making allowance for possible deviation from natural-response times in animals kept in the laboratory for shorter or longer times previous to physiological experiments.

Next, in field studies we examined the interactions between water flow, near-bed phytoplankton biomass and valve gape opening-closing response times of mussels in a dense bed. The study site, the narrow inlet to a shallow fjord, was chosen because of a predictable water-flow regimen created by the tide. Because of a high area-specific population filtration rate, a considerable variation in the chlorophyll *a* concentration could be expected within a half-tidal period. Therefore, the mussels are likely to be wide open during strong-flow periods with ample supply of phytoplankton, in contrast to periods with temporary tidal slacks and low supply rates of phytoplankton, where reductions in valve-gapes could be expected.

### MATERIALS AND METHODS

#### Laboratory Studies

Blue mussels, *Mytilus edulis*, were collected in the southeastern part of Kerteminde Fjord (Fyn, Denmark) in August 2003, January

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and May 2004, and brought to the nearby Marine Biological Research Centre where all experiments were conducted. In feeding experiments, mussels in aerated aquaria were offered suspended algal cells *Rhodomonas* sp. (almost spherical, about 6.3  $\mu\text{m}$  in diameter) that are 100% efficiently retained by the gills of *M. edulis* (Möhlenberg & Riisgård 1978). The algal cell concentration was measured by means of an electronic particle counter (Elzone 5380) by taking 15 mL water samples for analysis. All experiments were conducted at naturally low algal concentrations between 1,000–4,000 *Rhodomonas* sp. cells  $\text{mL}^{-1}$ , equivalent to about 1–5  $\mu\text{g chl } a \text{ L}^{-1}$  (Riisgård et al. 2003). In short-term laboratory experiments, the valve gapes of mussels were monitored by means of a digital camera taking a picture every 10 min. In long-term laboratory experiments, the valve gapes were monitored by means of an underwater video camera (SONY TRV25) recording the mussels for 2 s every 5 min. The individual valve gape was measured as the distance between the shells at the center of the exhalant opening and subsequently expressed as percentage of maximum distance recorded.

To study whether the opening-closing responses of the mussels and their duration are dependent on preceding feeding conditions a number of feeding/starvation experiments were performed. In one series of experiments, *Mytilus edulis* (Group #1, Table 1) were placed in an aerated aquarium with 1.2- $\mu\text{m}$  filtered seawater (20 psu) and the temperature was kept constant (Hetofrig cooler/heater) at the natural (field) acclimation temperature of the mussels (19.6°C). Afterwards the mussels were fed about 3,500 *Rhodomonas* sp. cells  $\text{mL}^{-1}$  for 2 h. The following day (Day 1) *Rhodomonas* sp. cells were repeatedly added to the aquarium and filtration rate measurements were performed under simultaneous monitoring of the valve gape of the mussels during the opening phase (0% to 80% of maximum registered valve gape during the whole experiment). After 5 h feeding, the mussels were separated into two groups (Group #2A and Group #2B, Table 1) and placed in two aerated aquaria with filtered seawater and constant temperature (19.6°C). For the next 4 days (Day 2 to Day 5) filtration rate measurements were performed while simultaneously monitoring the valve gapes of mussels in Group #2A, which were fed *Rhodomonas* sp. cells for about 5 h  $\text{d}^{-1}$ . Group #2B was starved in the same period. On Day 6 the valve-gape opening response was recorded in both Group #2A and Group #2B. *Rhodomonas* sp. cells were repeatedly added to the two aquaria with the two groups, and the valve gapes were monitored until all mussels were maximally open. Then the algal supply was subsequently stopped and the valve-gape closing response to decreasing algal concentration (<1000 cells  $\text{mL}^{-1}$ ) was recorded. When the mussels had reduced the algal concentration below the detection level of the electronic particle counter (about 100 *Rhodomonas* sp. cells  $\text{mL}^{-1}$ ; Riisgård

et al. 2003) further water sampling for particle analysis was ceased whereas the monitoring of valve gape continued until all mussels had closed their valves. During the following 13–14 days both groups of mussels starved whereupon, on Day 20 and Day 21 for Group #2A and Group #2B respectively, the valve-gape opening closing response was again registered.

In another series of experiments, the valve gape opening-closing response of mussels caused by presence/absence of algal cells was also recorded during periods with continuous algal supply followed by starvation periods without algal supply. The experimental period lasted for 68 and 50 days for Group #3 and Group #4 respectively (Table 1). Supply of algal cells was established from Day 1 to Day 38 for Group #3, and from Day 1 to Day 28 for Group #4, whereas long-term starvation was established from Day 38 to Day 68 for Group #3, and from Day 28 to Day 50 for Group #4.

Filtered seawater was continuously flowing through a strongly aerated aquarium with mussels. A dosing pump ensured constant addition of *Rhodomonas* sp. so that a well-defined steady-state level, corresponding to a natural phytoplankton concentration of 1.3–2.5  $\mu\text{g chl } a \text{ L}^{-1}$  was established (cf. Riisgård & Randlov 1981, Clausen & Riisgård 1996). The valve opening-closing response to presence/absence of algal cells was studied by stopping the dosing pump and the through-flow of seawater. The subsequent decrease in algal concentration caused by the filtering mussels was measured until the concentration was reduced to about 500 cells  $\text{mL}^{-1}$ . Then, by means of the dosing pump, this concentration level was maintained until all mussels had noticeably closed or reduced their valves. The duration of this closing response was defined as the time from when the concentration was 800 *Rhodomonas* sp. cells  $\text{mL}^{-1}$  (fully open mussels) to the time when the mean valve gape was reduced to 50% of the maximum registered during the preceding steady-state period. Then subsequently, the feeding conditions were resumed by switching on the algal dosing pump and the through-flow of filtered seawater and the immediate valve gape opening response recorded. The duration of the opening response was defined as the time from addition of algal cells after a short starvation period until the mean valve gape of the mussels reached 80% of the valve gape registered during the preceding steady-state. The long-term feeding period was succeeded by a starvation period to reveal if the mussels had changed the duration of the opening/closing response. To check this, the algal dosing pump was switched on for some hours to re-establish steady-state before the valve gape opening/closing response was recorded as previously described.

The experimental periods with added algal cells resulted in some growth of the mussels (increase in shell length of 0.2 and 0.7 mm in Group #3 and Group #4 respectively) and thus in an increased filtration rate, but a constant steady-state algal concentration was ensured by adjusting the dosing pump. Shell length and flesh dry weight (24 h, 110°C) were determined for all mussels after the experiments (Table 1).

#### Field Studies

The mussels, *Mytilus edulis*, studied in the present work formed a dense bed in the narrow inlet of Kerteminde Fjord, where a semidiurnal tide (amplitude  $\pm 0.2$  m) forces a strong, alternating in- and outgoing current (Fig. 1). In 2002 the mussel bed was overgrown by dense macro algae (*Fucus* sp.).

During the summer of 2002, simultaneous registrations of wa-

TABLE 1.

*Mytilus edulis*. Data for groups of mussels at the end of experiments. Number of individuals ( $n$ ), shell length ( $L$ ), body wet- and dry weight ( $W_w$ ,  $W_d$ ).

Group #	$n$	$L$ (cm $\pm$ SD)	$W_w$ (g $\pm$ SD)	$W_d$ (g $\pm$ SD)
1	13	3.997 $\pm$ 0.106		
2A	7	3.990 $\pm$ 0.080	1.958 $\pm$ 0.213	0.334 $\pm$ 0.029
2B	6	3.960 $\pm$ 0.139	2.036 $\pm$ 0.475	0.342 $\pm$ 0.104
3	11	4.191 $\pm$ 0.112	2.671 $\pm$ 0.500	0.462 $\pm$ 0.110
4	19	2.702 $\pm$ 0.195	0.414 $\pm$ 0.071	0.070 $\pm$ 0.015



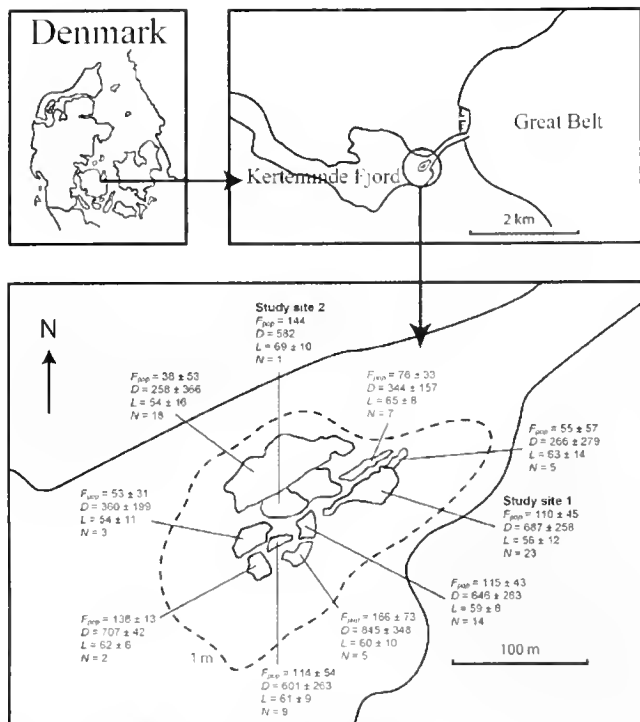


Figure 1. Mussel bed and the 2 study sites in the inlet to Kerteminde Fjord.  $D$  = mean density (ind.  $m^{-2}$ );  $L$  = mean shell length (mm);  $F_{pop}$  = calculated mean area-specific population filtration rate ( $m^3 m^{-2} d^{-1}$ ) of the mussels;  $N$  = number of sampling sites,  $\pm$ SD is indicated.

ter elevation, current speed, chlorophyll *a* (chl *a*) concentration and valve gapes of mussels were carried out as 10 one-day studies at different selected locations on the shallow north-western part (Site 1: field-study days #1 to #6, and #10), and in the somewhat deeper middle part (Site 2: field-study days #7, #8, and #9, see Fig. 1) of the mussel bed. The water elevation was registered in the fjord-inlet about 400 m from the mussel bed by means of a CTD (Aquamat). In all cases, the current speed, chl *a* and valve gapes were registered on the same spot (with half a square meter) on the selected locations. The current speed was registered by means of a current propeller (Hydrobios) fixed at 20-cm height Site 1 and 50-cm height above the mussel bed at Site 2. The chl *a* (chl *a*) concentration was registered simultaneously at 2 to several heights above the mussel bed with sampling intervals from 10–60 min. The lowest sampling height always equaled 1-cm height above the mussel bed. Additional heights were up to 70 cm above the bed, depending on the study location. Sampling heights and intervals have been specified for each field-study day on the prepared figures of chl *a* contours. The water samples (50 mL) were taken manually by means of syringes attached to silicone pipes with endings through small holes in a rigid plastic pipe fixed in the bottom. Before sampling, the tubes were emptied of the “old” water (water from the prior sampling) to prevent mixing of new with old water. The water samples were immediately filtered on a GF/C filter (pore size = 1.2  $\mu m$ ) followed by 20 h of chl *a* extraction in 10 mL 96% ethanol. The fluorescence of the supernatant was measured on a fluorometer (Turner, model 450) and the fluorescence values were converted into concentration of chl *a* by means of a regression function determined for each day of study. The measured chlorophyll *a* concentrations represent time-averages over 5–6 min (= the water-sampling time).

In each field study a group of undisturbed mussels (from 4–10 individuals) was photographed every 5 min by means of a time-lapse video camera (Sony TRV 25) installed in a housing (Ikelite) fixed on a heavy tripod. The pictures (resolution:  $480 \times 640$  pixels<sup>2</sup>) were analyzed for the valve gapes (i.e., the distance between the valves) of the mussels. For each particular mussel the valve gapes were standardized to the observed maximal valve gape of the mussel during the particular time-series. Hence, a valve-opening degree of 100% represents the maximal valve gape of the mussel. An uncertainty test making repeated measurements of the same distance revealed a measuring uncertainty for the valve gape analysis between 6% and 28% (the larger the distance measured, the less the uncertainty). Consequently, the results from the deeper part of the mussel bed, where the mussels were registered with less camera zoom, are expected to be more uncertain. Minor uncertainties in valve gapes are caused by smaller variations in the orientations of the mussels to the camera during recording.

Prior to the field observations, mussel density ( $D$ , ind.  $m^{-2}$ ), mean shell length ( $L$ , mm), and area-specific population-filtration rate of the mussel bed ( $F_{pop}$ ,  $m^3 m^{-2} d^{-1}$ ) were determined and the patchy distribution of mussels was mapped using Global Positioning System (GPS).  $F_{pop}$  was estimated as:  $F_{pop} = \sum F_{ind}/A$ , where  $F_{ind}$  ( $l h^{-1}$ ) =  $0.0012L^{2.14}$  (Kjørboe & Møhlenberg 1981, corrected by Riisgård 2001a) and  $A$  = the area investigated for mussels. Knowing the water depth ( $H$ , m) and assuming complete mixing of the water column, the half-life time of phytoplankton above the mussel bed was calculated as:  $t_{1/2} = H \times \ln 2 / F_{pop}$ .

The test statistics (significance level  $\alpha = 0.05$ ) have been calculated in Microsoft Excel 2003 and compared with critical values from Zar (1999).

## RESULTS

### Laboratory Studies

Figure 2 shows an example of valve opening response (during the first 15 min) to an addition of algal cells to a group of starved mussels, and further, after a period with a maintained algal concentration between 1000 and 5000 cells  $mL^{-1}$  to ensure maximum valve gape, the figure shows the closing response to a decreasing algal cell concentration. Figure 3 shows similar valve closing responses caused by low algal concentrations in two experiments. In the first experiment (Fig. 3A), a group of mussels was initially fed for 6 days before recording of the valve-gape closing response and then subsequently starved during the following 20 days. Initially the mean valve gape decreased nearly linearly, but the closing response was faster in fed than in starved mussels. In the second experiment (Fig. 3B), the group of mussels was starving during the whole experimental period. Both after 6 and 21 days of starvation the mean valve gape initially decreased nearly linearly, but the closing response was slower in the mussels starved for 21 days.

Figure 4 shows the opening response of mussels to addition of algal cells at time zero. It is seen that the opening time depends on the preceding feeding conditions in such a way that starved mussels seem to have a longer opening time than previously fed mussels. The opening response time for mussels fed daily with algal cells is shown in Figure 5. During the algal exposure period of 6 days, the duration of the opening response is decreasing, whereas, after a subsequent starvation period of 20 days, the opening response time had increased. The observed valve gape responses to presence/absence of algal cells in the ambient water shows that the

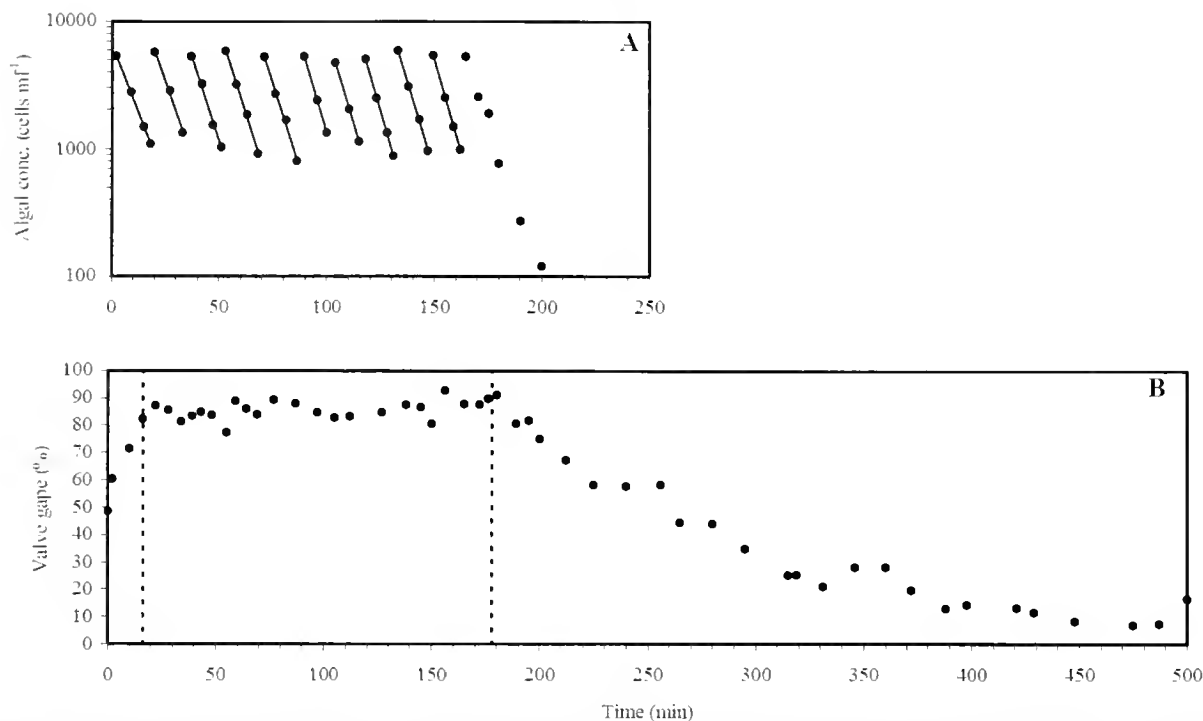


Figure 2. *Mytilus edulis*. (A) Exponential decrease in algal cell concentration after repeated additions of algal suspension to a well-mixed aquarium with actively grazing mussels (Group #2A). Slope of regression lines represent filtration rates. (B) Mean valve gape of the mussels. Opening response (0–16 min), constant filtration rate and maximal valve gape (16–178 min), closing response (178–315 min).

valve opening/closure responses are strongly influenced by the preceding feeding conditions.

Valve-closing response were also studied in steady-state feeding experiments and Figure 6 shows that the duration of the closing response initially declined with algal exposure time to become near constant in the remaining period, whereas on the other way round, the duration of the closing response was increasing during the subsequent starvation period. Figure 7 shows a similar closing response, although less pronounced. The closing response is slowly decreasing with the algal exposure time from Day 1 to Day 18 to be constantly low (about 10 min) during the rest of the feeding period. In the subsequent starvation period (from Day 29 to Day 50), the duration of the closing response increased apparently linearly as a function of time at a rate of about 10 min d<sup>-1</sup>. The duration of the opening response (defined as the time from readdition of algal cells after a short period of valve closure caused by stoppage of the dosing pump until the mean valve gape had increased to maximum) is shown on Figure 8. It is seen that during the period in which the mussels were exposed to a constant steady-state algal cell concentration (from Day 1 to Day 38), the duration of the opening response was about 90 min, but in the subsequent starvation period (from Day 39 to Day 68) the opening-response time decreased to about 20 min. The previous observations indicate that the period during which mussels may “learn” or “forget” to respond to the presence/absence of algal cells can last for weeks.

Mussels that were maximally open and actively filtering at a low steady-state concentration of about 700 *Rhodomonas* sp. cells mL<sup>-1</sup> responded to a slight reduction in the algal concentration by a nearly immediate reduction in valve gape (Fig. 9). From this observation it can be concluded that the critical algal concentration below which the mussels close their valves is only slightly lower than 700 *Rhodomonas* sp. cells mL<sup>-1</sup>, or 0.9 µg chl *a* L<sup>-1</sup>.

#### Field Studies

The density, mean shell length and calculated area-specific population-filtration rate ( $F_{pop}$ ) of the mussel bed are shown in Figure 1.  $F_{pop}$  ranged between 38 and 166 m<sup>3</sup> m<sup>-2</sup> d<sup>-1</sup>, with a local maximum of 255 m<sup>3</sup> m<sup>-2</sup> d<sup>-1</sup> (not shown). Assuming a maximal water depth of 1 m and complete water mixing, the calculated half-life time of phytoplankton above the mussel bed is between 6 and 26 min. Because complete water mixing may never be attained in the benthic boundary layer, the actual half-life of phytoplankton in the near-bed water is likely to be even shorter. Hence, a considerable depletion in near-bed chl *a* concentration was expected on the mussel bed around tidal slacks.

The time-series of water depth, current speed (averaged over 5–10 min), chl *a* concentration, and valve-opening degrees of the mussels from the 10 field-study days are depicted in Figure 10. The water depth and current speed both followed the semidiurnal tide. The tidal range was up to about 0.4 m and the current speed ranged between tidal maxima of up to 0.4 m s<sup>-1</sup> at 20 cm above the mussel bed and zero when the tide changed between in- and outgoing flow. In general, the measured variables showed tidal variation. The transition from strong to zero tidal flow was recorded on field-study days #1 to #3 (Fig. 10). It is seen that the mussels were wide open in the initial phase of strong tidal flow with near-bed chl *a* concentrations above 1 µg L<sup>-1</sup>, whereas around tidal slack, where the near-bed phytoplankton biomass was grazed below about 1 µg chl *a* L<sup>-1</sup>, the mussels reduced their valve gapes. A time lag in the response to low phytoplankton concentrations may be expected, which may explain why (e.g., the mussels in the first 15–30 min of field-study day #3) are fully open even though the chl *a* concentration is extremely low. Likewise, maintained maximal valve-opening degrees are observed during strong tidal flow

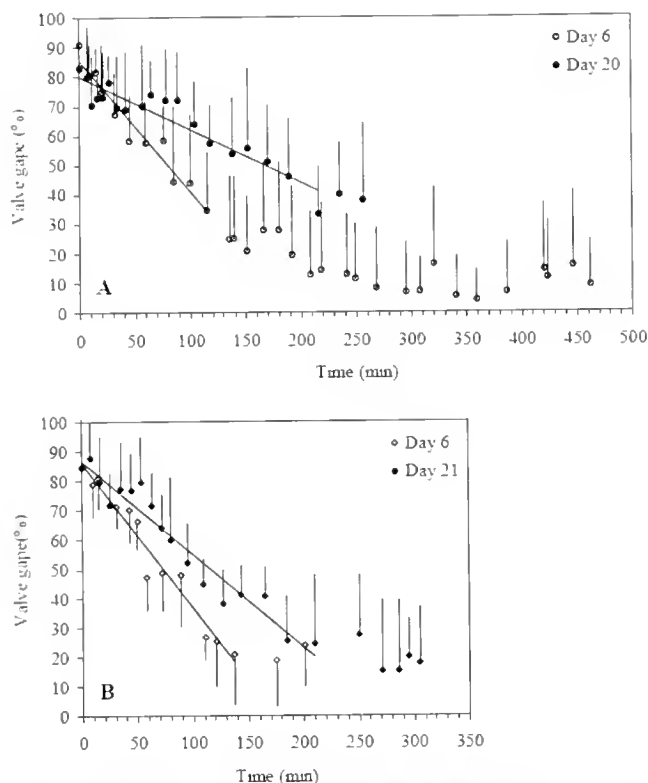


Figure 3. *Mytilus edulis*. Valve-gape closing response in short-term algal exposure experiments in which 0 min is the time when the decreasing algal concentration in the aquarium with filtering mussels passed  $1,000$  *Rhodomonas* sp. cells  $\text{mL}^{-1}$ . (A) Reduction of the mean valve gape (VG, %  $\pm$  SD) as a function of time ( $t$ , min) in Group #2A on Day 6 ( $\square$ ) and Day 20 ( $\bullet$ ). The initial reduction in valve gape could be expressed by the inserted regression lines:  $VG = -0.443t + 85.4$  ( $r^2 = 0.946$ ) and  $VG = -0.179t + 80.0$  ( $r^2 = 0.880$ ) for Day 6 and Day 20 respectively. (B) The initial reduction of valve gape recorded in Group #2B on Day 6 ( $\diamond$ ) and Day 21 ( $\blacklozenge$ ) could be expressed by the inserted regression lines as:  $VG = -0.505t + 86.6$  ( $r^2 = 0.949$ ) and  $VG = -0.316t + 86.7$  ( $r^2 = 0.939$ ) for Day 6 and Day 21 respectively.

on field-study day #4 for most of the mussels. During field-study days #5 and #6 it was observed, as the tidal flow reincreased after tidal slack, that the near-bed chl  $a$  concentration increased above  $1 \mu\text{g L}^{-1}$ , whereas at the same time the mussels reincreased their valve-opening degrees to maximum. The same pattern with temporary reductions in mussel valve gapes during tidal slack appears from the field-study days #7, #8, and partly field-study day #9, carried out on the deeper part of the mussel bed (Fig. 1, Study site 2). More scatter in the depicted valve-opening degrees is expected from these 3 field-study days because of a higher uncertainty in the valve-gape analysis. However, on field-study day #9 the high degree of scatter in valve gapes and resultant low mean valve-opening degree seem to be markedly pronounced (even at high tidal flow rates) coincident with a constant exposure to extremely low near-bed chl  $a$  concentrations (below  $1 \mu\text{g L}^{-1}$ ). Hence, short-term individual variability in valve valve-gape may be an indicator of shorter starvation periods. The extremely low chl  $a$  concentrations registered on field-study day #9 may be explained partly by incoming low chl  $a$  concentrations from the seaside and/or partly by limited replenishment of phytoplankton above the mussel bed because of a small tidal range this day. To this end, the results from field-study day #10 show an atypically valve-gape behavior. In

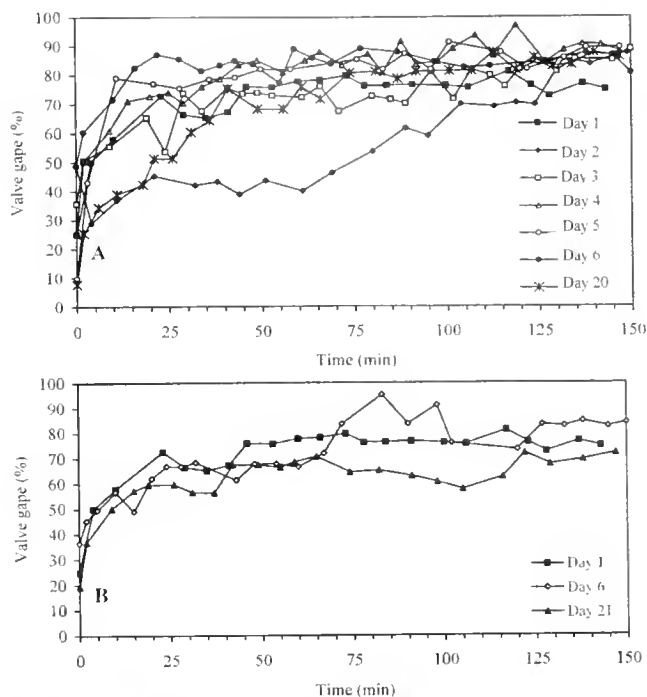


Figure 4. *Mytilus edulis*. Opening response of mussels in short-term experiments. (A) Mean valve gape as a function of time after algal addition to aquarium at time zero for mussels in Group #1 and Group #2A on Day 1, 2, 3, 4, 5, 6, and Day 20. (B) Mean valve gape for mussels in Group #1 and Group #2B on Day 1, 6, and Day 21 after addition of algal cells at time zero.

particular, for one of the mussels a prolonged (150 min) valve-gape reduction phase is initiated at a high tidal flow rate and chl  $a$  concentrations well above  $1.5 \mu\text{g L}^{-1}$ . It is not clear whether the chl  $a$  concentration of the inhalant water of the particular mussel has been lower than the registered one at 1 cm above the mussel bed.

Figure 11 shows the ratio of near-bed to surface chl  $a$  concentration ( $C_b/C_s$ ) versus current speed (pooled raw data and data-fit). An analysis of variance ( $F$ -test, Zar 1999) showed a significant ( $P < 0.0005$ ;  $r^2 = 0.25$ ) data-fit to the following asymptotic regres-

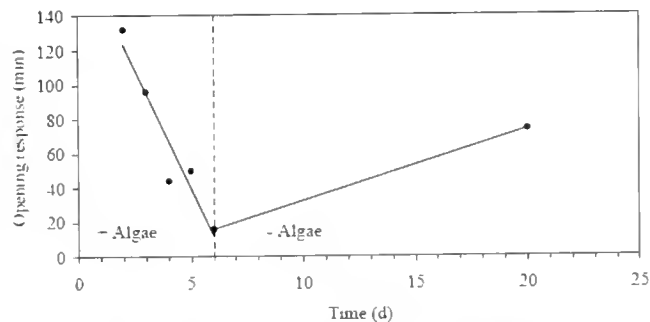


Figure 5. *Mytilus edulis*. Opening response for mussels (Group #2A) in short-term experiment. The duration of the opening response, defined as the time from start of experiment until mean valve gape was 80% of the maximum registered. When the mussels were exposed to algal cells the opening response ( $DO$ , min) was linearly decreasing with time ( $t$ , d). The inserted regression line for Day 1 to Day 6 (+algae):  $DO = -27.8t + 178.8$  ( $r^2 = 0.911$ ); line for Day 6 to Day 20 (-algae):  $OP = 4.14t - 8.86$ .

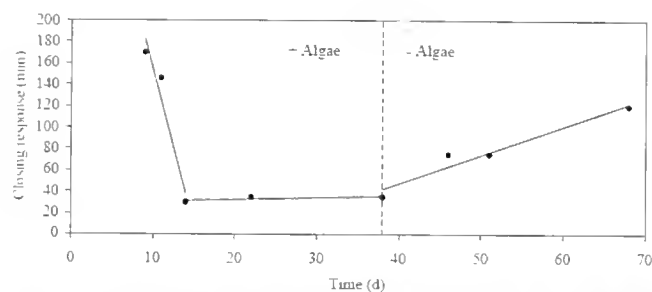


Figure 6. *Mytilus edulis*. Valve gape closing response for mussels (Group #3) in long-term experiments. The closing response as a function of time during which the mussels were first exposed to algae (Day 1 to Day 38) followed by a period without algae (Day 38 to Day 68). Trends have been expressed by inserted regression lines.

sion model:  $C_p/C_s = 1.794 - 1.147e^{-0.00104U/F_{pop}}$ , where  $U$  ( $m\ s^{-1}$ ) = the current speed and  $F_{pop}$  (the area-specific population-filtration rate of the mussel bed) =  $1.16 \times 10^{-3} m^3\ m^{-2}\ s^{-1}$ . Hence, the vertical chl  $a$  gradient above the mussel bed became steeper as the current speed decreased.

Figure 12 (left) shows the means of the time-averaged (1 h intervals were used to approach independence between pooled valve-gape data) valve-opening degrees in various tidal flow phases. On the basis of a nonparametric analysis of variance by ranks (Kruskal-Wallis test; Zar 1999) it is found that the mean valve-opening degrees were different between flow phases ( $P < 0.0005$ ). A subsequent nonparametric multiple comparison test using rank sums (Dunn test; Zar, 1999) shows that the mean valve-opening degrees observed in the flow phases  $t = -3, -2, -1$ , and 2 were not significantly different from each other, but the mean valve-opening degrees in the flow phases  $t = -3, -1$ , and 2 were significantly ( $0.005 < P < 0.01$ ) different from the reduced ones observed around tidal slack at  $t = 0$  and  $t = 1$ . The mean valve-opening degrees have also been plotted versus the corresponding mean time-averaged near-bed chl  $a$  concentrations in Figure 12 (right). It is seen that the mean near-bed chl  $a$  concentrations were generally low ( $< 1.6\ \mu g\ L^{-1}$ ) and that reduced valve-opening degrees associated with chl  $a$  concentrations below  $1.0\ \mu g\ L^{-1}$ . Table 2 shows a calculated "grazing index," chl  $a_b$ /chl  $a_a$ , where the underscores "b" and "a" refer to the time just before and right after a valve-gape opening-closing response, respectively. The index is significantly lower ( $0.005 < P < 0.01$ ) for the valve-closing phase

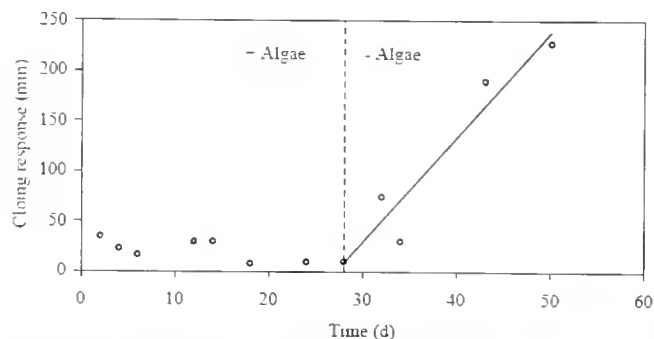


Figure 7. *Mytilus edulis*. Valve closing response for mussels (Group #4) in long-term experiments. The closing response as a function of time during which the mussels were first exposed to algae (Day 1 to Day 28) followed by a period without algae (Day 28 to Day 50). Trends have been expressed by inserted regression lines.

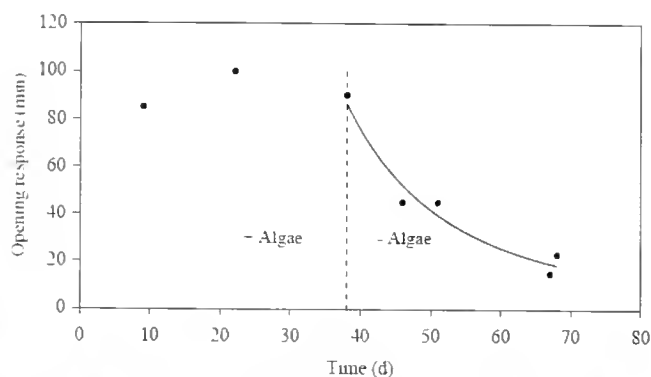


Figure 8. *Mytilus edulis*. Valve gape opening response for mussels (Group #3) in long-term experiment. The opening response ( $DO$ , min) as a function of time ( $t$ , d) during which the mussels were first exposed to algae (Day 1 to Day 38) followed by a period without algae (Day 38 to Day 68). For the period without algae, the decreasing opening response can be described by the equation:  $DO = 10^6 t^{-2.656}$  ( $r^2 = 0.922$ ).

compared with the valve-opening phase, which shows that the closing and opening phase occurred simultaneously with a decrease (grazing index  $< 1$ ) and increase (grazing index  $> 1$ ) in near-bed chl  $a$  concentration, respectively. The proposed causal relation between near-bed phytoplankton biomass (chl  $a$ ) and mussel valve gape was investigated further in a shallow tidal lagoon close to Kerteminde Fjord. Here, 4 mussels settled on the bottom and 2 mussels settled on a stone 16 cm above the bottom, respectively, were video-registered simultaneously. The current speed was below the detection level ( $= 0.05\ cm\ s^{-1}$ ) of the current propeller, but changes in the tidal flow was estimated visually by following small particles in the flow. The results have been shown in Figure 13. It is seen that the benthic concentration-boundary layer increased in thickness during the recording interval, along with a decrease in tidal flow rate (not shown). As the near-bottom chl  $a$  concentration became below  $0.8\ \mu g\ L^{-1}$ , the 4 mussels on the bottom reduced their valve gapes, whereas the 2 mussels on the stone higher up in the concentration boundary layer remained fully open.

Finally, the distributions of the valve-opening and closing times (taken from reduction of the valve-opening degree from 100% to 50%, and opening from 50% to 100%, respectively) have been

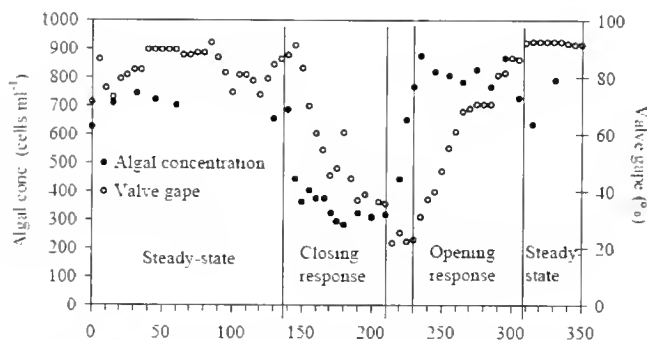


Figure 9. *Mytilus edulis*. Identification of critical algal concentration for stimulation of maximum valve gape in a group of mussels. At the initial steady-state algal concentration of about 700 *Rhodomonas* sp. cells  $mL^{-1}$  the mussels are maximally open, but reduction in algal concentration caused by temporary stoppage of algal dosing pump results in a closing response, that is followed by an opening response when the algal dosing pump is reactivated resulting soon after in a re-established steady-state where the mussels are again fully open.

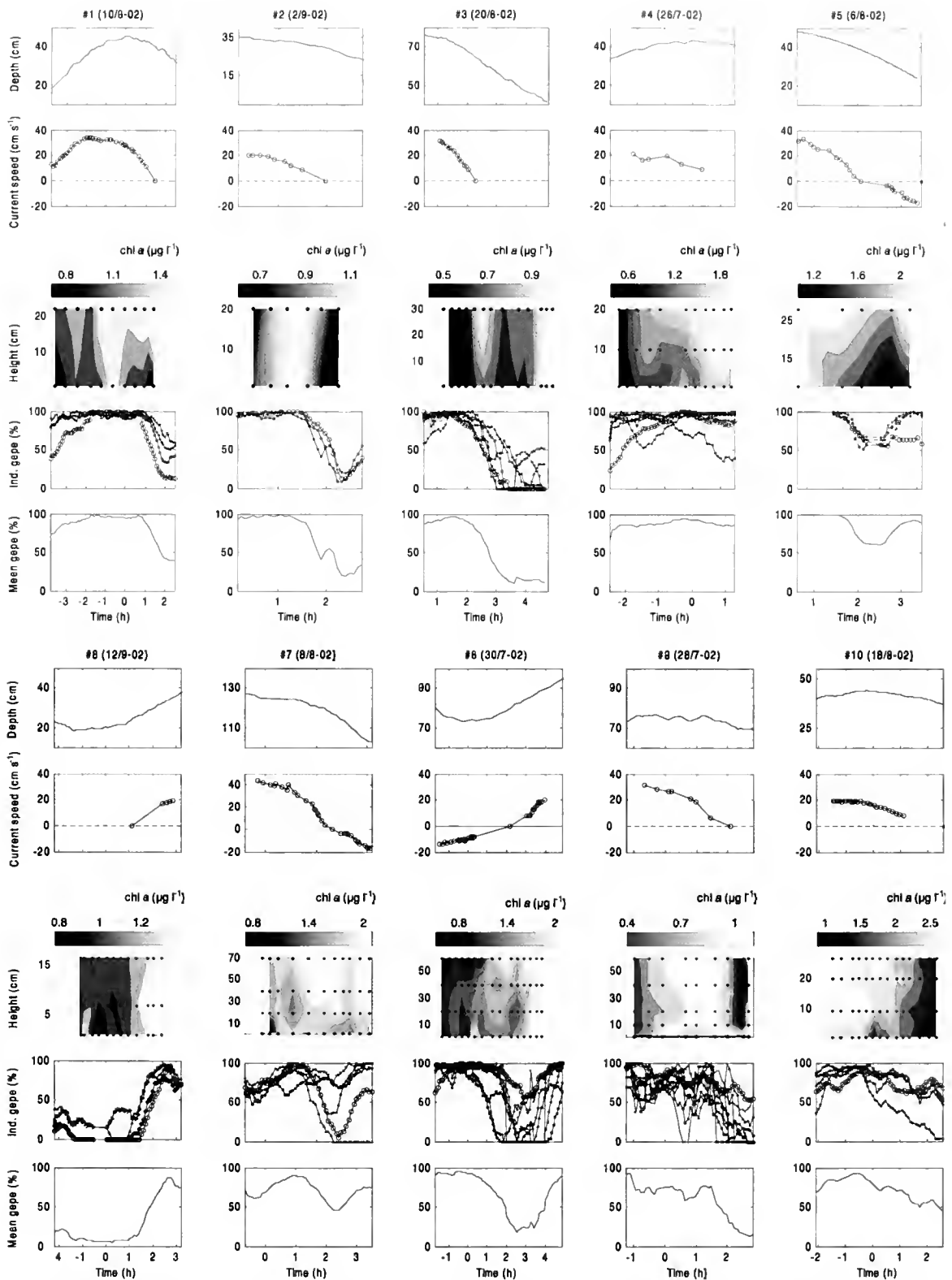


Figure 10. Field-study days #1 to #10 in Kerteminde Fjord: Top to bottom: Time-series of water depth above mussels, current speed, contours of chl *a* concentration ( $\mu\text{g L}^{-1}$ ) above the mussel bed (sampling time and heights above the mussel bed marked with dots), individual percentage valve-opening degree, and mean percentage valve-opening degree of the mussels. Current speeds above and below zero indicate in- and outgoing tidal flow, respectively. Height 1 cm in the chl *a* contours represents 1 cm above the mussels.

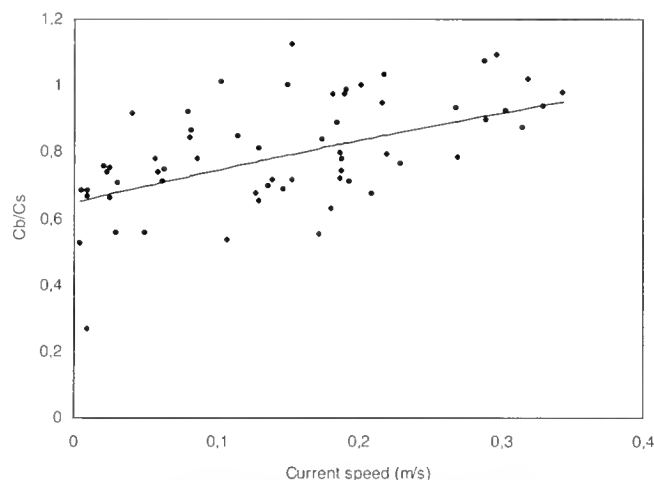


Figure 11. Pooled data of the depletion index ( $C_p/C_i$ ) versus tidal current speed. Least squares asymptotic fit of data ( $r^2 = 0.25$ ;  $P < 0.0005$ ):  $C_p/C_i = 1.794 - 1.147e^{-0.00104U/F_{pop}}$ , where  $U$  ( $m\ s^{-1}$ ) = the current speed, and  $F_{pop}$  (the area-specific population-filtration rate of the mussel bed)  $= 1.16 \times 10^{-3}\ m^3\ m^{-2}\ s^{-1}$  ( $= 100\ m^3\ m^{-2}\ d^{-1}$ )

shown in Figure 14. Table 2 shows that the mean duration of the mussel closure phase were almost of same duration: 50 and 59 min, respectively.

## DISCUSSION

### Laboratory Studies

In a recent work Riisgård et al. (2003) studied the valve opening-closing phenomenon and response times in three species of filter-feeding bivalves (*Cardium edule*, *Mytilus edulis*, *Mya arenaria*) in the presence and absence of algal cells in controlled laboratory experiments. Opening state and correlated filtration rate at varying or maintained levels of algal concentration was studied in filtration rate experiments combined with video observation. When initially unfed bivalves were offered algal cells, the animals soon opened their siphons/valves simultaneously with a pronounced increase of the filtration rate. On the other hand, when open and actively filtering bivalves experienced decreasing algal

concentrations below a certain level, this lead within a few hours to a reduced opening state and cessation of filtration activity. However, a considerably shorter valve-closure response time of only 15 min was recorded by Riisgård & Randlov (1981). The short response time may be caused by the experimental conditions because the valve-gape observations were made as part of a long term (47 d) feeding experiment imitating realistic, natural food concentrations. This suggestion is in agreement with the present observations.

This study shows that the duration of the well-known valve-gape response of mussels to the presence or absence of algal cells in the ambient water (Riisgård et al. 2003) is strongly influenced both by the preceding feeding conditions and the length of starvation. The period during which mussels may "learn" or "forget" to respond to the presence or absence of algal cells has been found to last for weeks in mussels kept in the laboratory. This knowledge is relevant for a correct understanding of the frequently used term "acclimation" (e.g., acclimation of experimental mussels to different laboratory conditions) such as changes in temperature, salinity or feeding conditions in experimental set-ups or storage tanks with or without through-flowing seawater, see also Kittner & Riisgård (2005). Many parameters (e.g., oxygen uptake rate, filtration rate, elimination- and uptake rates of pollutants) measured in different studies using mussels as experimental animals are strongly dependent on the valve-opening degree, and thus—although indirectly—the preceding feeding conditions or length of starvation after being collected in the field. Newly collected mussels may react very differently to experimental conditions compared with long-term laboratory "acclimated" mussels.

Valve closure and cessation of filter feeding in mussels at algal concentrations below a certain trigger level has been designated "lower trigger-level cessation" by Riisgård (2001b). In the present work, this critical algal concentration has been determined to be about  $0.9\ \mu g\ chl\ a\ L^{-1}$  in quite good agreement with a suggested lower trigger-level of about  $0.5\ \mu g\ chl\ a\ L^{-1}$  (Clausen & Riisgård 1996, Dolmer 2000a, 2000b). The valve-closing phenomenon, and subsequent reduction of the metabolism (Jørgensen et al. 1986), seems to represent an adaptation to filter feeding in extremely meager situations as discussed by Riisgård (2001b). From the present laboratory findings it seems likely that the valve opening/

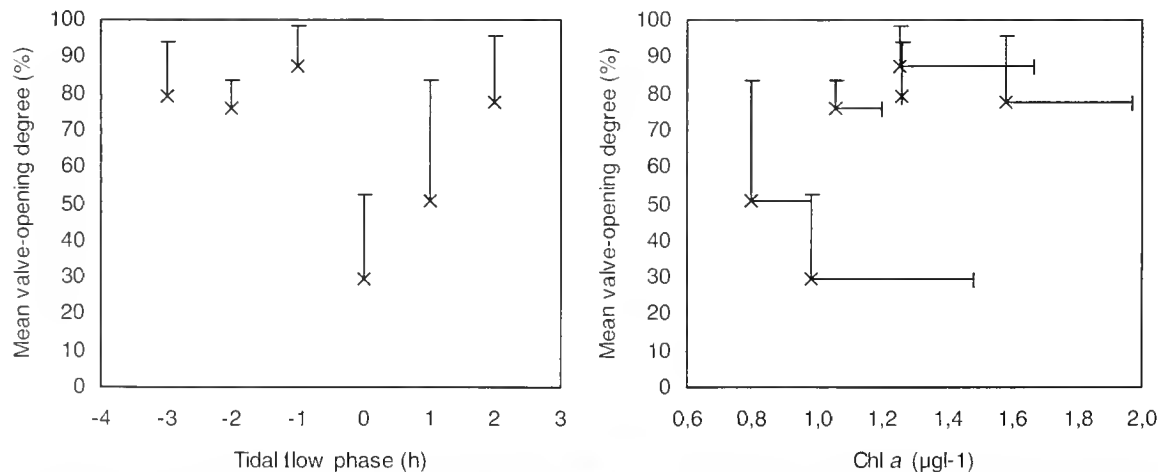


Figure 12. Left: Mean time-averaged (1 h) valve-opening degree of mussels (+SD) in various tidal flow phases, indicated as 1-h time intervals up to (–) and after (+) zero flow. Right: Mean time-averaged (1 h) valve-opening degree of mussels (+SD) versus corresponding mean time-averaged near-bed chl *a* concentrations (+SD).

TABLE 2.

*Mytilus edulis*. Mean duration  $\pm$  SD of valve gape closing phase (taken as the duration from valve-opening degree 100% to 50%) and opening phase (taken as duration from valve opening degree 50% to 100%). Mean grazing index:  $(\text{chl } a_p/\text{chl } a_a)_{\text{mean}}$ , where  $\text{chl } a_p$  = the near-bed chlorophyll *a* concentration right before initiation of the valve-gape response and  $\text{chl } a_a$  = the near-bed concentration right after initiation of the valve-gape response. *N* = sample size.

	Closure Phase	Opening Phase
Mean duration $\pm$ SD (min)	59 $\pm$ 22 ( <i>N</i> <sub>mussel</sub> = 26)	50 $\pm$ 19 ( <i>N</i> <sub>mussel</sub> = 13)
Mean (chl <i>a</i> <sub>p</sub> /chl <i>a</i> <sub>a</sub> ) $\pm$ SD	0.8 $\pm$ 0.2 ( <i>N</i> <sub>chl <i>a</i></sub> = 6)	1.2 $\pm$ 0.1 ( <i>N</i> <sub>chl <i>a</i></sub> = 4)

closing-response times in the field may be considerably faster than usually observed in the laboratory, a suggestion that has been confirmed by the present field studies.

#### Field Studies

Filter-feeding mussels are important sinks for phytoplankton in shallow fjords and coastal waters. In such places dense mussel beds with area-specific population filtration rates of 100–200 m<sup>3</sup> m<sup>-2</sup> d<sup>-1</sup> may exert a considerable grazing impact (Jørgensen 1984, 1990), as also confirmed by the present work (Fig. 1). The phytoplankton biomass in areas with mussel beds may be significantly reduced, and a pronounced near-bottom concentration-boundary layer may develop (Fréchette et al. 1989, Prins et al. 1998, Norén et al. 1999, Dolmer, 2000a, Tweddle et al. 2005). The steepness of horizontal and vertical gradients in the phytoplankton biomass in such a boundary layer is determined by current speed and turbulent mixing of the ambient water (van Duren 2005, Lassen et al. 2006). Consequently, mussels in dense beds may experience a dynamic nutritional environment as also demonstrated in the present work. The results shown in Figure 11 agree with earlier findings that mussels within a mussel bed become increasingly limited in their supply of phytoplankton at decreasing current speeds caused by steeper horizontal and vertical phytoplankton gradients above the mussel bed (Fréchette et al. 1989, Dade 1993, Butman et al. 1994, van Duren et al. 2005, Lassen et al. 2006, Tweddle et al. 2005). The tidal flow rate clearly had an effect on the valve-gape behavior of the mussels (Fig. 11), but the present study suggests this effect to be an indirect one, because the mussels (located between dense macro-algae, *Fucus* sp.) were never exposed to such high current speeds reported to affect the valve gape of mussels (see e.g., Newell et al. 2001). As pointed out by Yu & Culver (1999), local zones of depleted water may be found inside dense mussel beds and this phenomenon is likely to have caused the observed “atypical” behavior of the mussels on field-study day #10 (Fig. 10).

Prevailing phytoplankton concentrations in noneutrophicated waters usually lie between 1 and 5  $\mu\text{g}$  chl *a* L<sup>-1</sup>, and in this concentration interval the blue mussel is wide open and filtering at maximum rate (Riisgård 2001a, Riisgård et al. 2003). However the phytoplankton biomass in the near-bottom concentration boundary layer is often substantially lower. Thus, Dolmer (2000a) measured the vertical distribution of phytoplankton above *Mytilus edulis* beds in Limfjorden, Denmark. In one case, the chl *a* concentration measured 2 m above the mussel bed (3.2  $\mu\text{g}$  L<sup>-1</sup>) declined towards the bottom to about 0.3  $\mu\text{g}$  L<sup>-1</sup>, which is less than the threshold

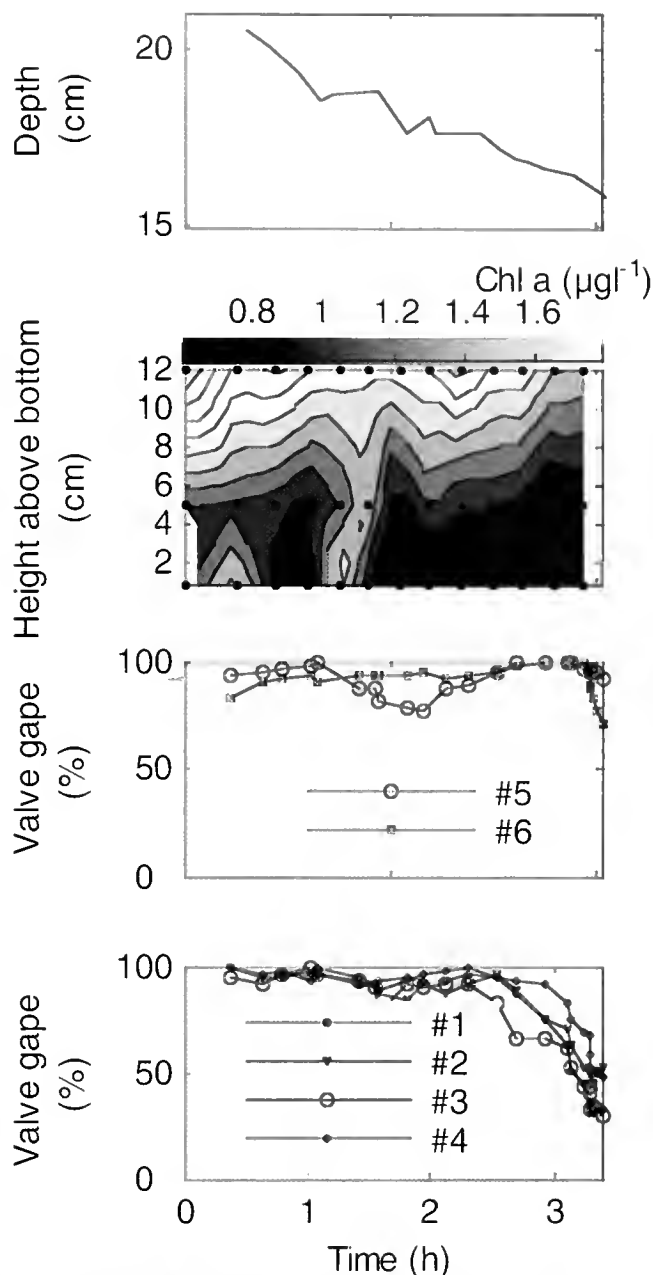


Figure 13. Field study in shallow lagoon close to Kerteminde Fjord. Time-series of water depth, contours of chl *a* concentration ( $\mu\text{g}$  L<sup>-1</sup>) (sampling time and heights above the mussel bed marked with dots), individual percentage valve-opening degrees of mussel #5 & #6 placed on a stone 15 cm above the bottom, individual percentage valve-opening degrees of mussel #1 to #4 placed on the bottom. Height zero in the chl *a* contours represents 1 cm above the bottom.

value (0.7–1.0  $\mu\text{g}$  L<sup>-1</sup>) below which the blue mussel closes its valves (Clausen & Riisgård 1996, Dolmer 2000a; b, Newell et al. 2001, Kittner & Riisgård 2005). As stated by Riisgård (2001b) the valve-closing phenomenon, which has also been clearly demonstrated in the present work, represents a physiological adaptation to filter feeding in extremely meager situations, when a reduced valve gape reduces the metabolism of the mussel (Jørgensen et al. 1986).

Tidally induced variation in bivalve filtration activity (valve gape and/or siphon opening degree) has earlier been observed in

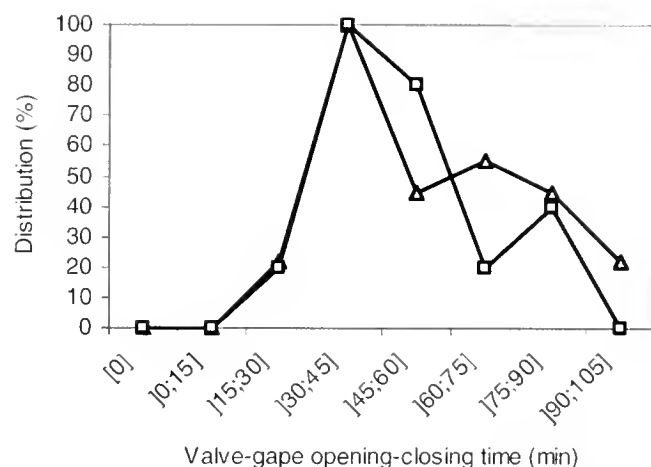


Figure 14. Percentage distributions of valve-gape opening (squares) and closing (triangles) times, taken as the duration from valve-opening degree 50% to 100% and 100% to 50%, respectively.

*situ* in *Mya arenaria* (Roegner 1998, Thorin et al. 1998), *Mytilus edulis* (Newell et al. 1998, 2005), and *Venerupis corrugatus* (Stenton-Dozey & Brown 1992). Newell et al. (1998) registered coincidence between reduced valve gapes in *M. edulis* and low particle concentrations in during the tidal period, but the observed responses in valve gape/siphon opening were not directly correlated to tidally driven variation in phytoplankton biomass in the near-bottom water. Recently, Newell et al. (2005) registered two peaks in exhalant opening diameter of *M. edulis* in a dense bed: a peak when new water with food particles was brought in by the tide, and a peak during ebb, coincident with a high settling flux of marine snow. In the remaining tidal period the mussels had reduced valve gapes. The present study shows that the valve gape of mussels is maximum during tidal phases with sufficiently high flow and near-bed chl *a* concentrations above roughly  $1 \mu\text{g L}^{-1}$ , but at slower flow rates and chl *a* concentrations  $<1 \mu\text{g L}^{-1}$  the mussels reduce their valve gape. The present observations indicate

that the mussel *in situ* valve gape opening-closing response to variation in phytoplankton biomass is short (50 min) compared with the response times in unfed mussels in the laboratory (up to 180 min) (Riisgård et al. 2003), but compare well with fed mussels in the present laboratory studies and with results from a field-study conducted by Newell et al. (1998). It can be read from Figure 6 in Newell et al. (1998) that the closure response of *M. edulis* in Mud Cove (Maine, USA) was about 60 min, whereas the subsequent opening phase was slightly shorter (about 40 min).

## CONCLUSION

Laboratory studies of valve-gape response of mussels to presence or absence of algal cell have demonstrated that valve opening and closing response times are strongly influenced by the preceding feeding conditions. The period during which mussels may "learn" or "forget" to respond to the presence or absence of algal cells can last for weeks in the laboratory. Field studies have revealed that phytoplankton concentrations higher than about  $1 \mu\text{g chl } a \text{ L}^{-1}$  stimulate the mussels to keep their valves wide open, whereas lower near-bed concentrations cause valve closure, or reduced valve gape. The *in situ* valve opening and closing times in response to variation in near-bed chl *a* concentration are considerably faster than in unfed mussels in the laboratory. This knowledge is essential for making allowance for deviation from natural response times in mussels kept in the laboratory previous to experimental physiological studies. Further, the opening/closing response times to presence/absence of phytoplankton may influence the actual grazing impact of mussels, and therefore, awareness of response times may be of importance for future modeling because of hysteresis that can mask the connection between filter-feeding activity and the concentration of phytoplankton.

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## GROWTH, PRODUCTION AND ECONOMIC EVALUATION OF EARTHEN PONDS FOR MONOCULTURE AND POLYCULTURE OF JUVENILES SPOTTED BABYLON (*BABYLONIA AREOLATA*) TO MARKETABLE SIZES USING LARGE-SCALE OPERATION

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**ABSTRACT** The study was conducted to determine the feasibility for growing-out of the spotted babylon juveniles (*Babylonia areolata*) to marketable sizes with the large-scale operation of 20.0 × 20.0 × 1.5 m earthen ponds at Petchaburi province, Thailand. The production and economic analysis for monoculture of spotted Babylon were compared with the polyculture with sea bass (*Lateolabrax japonicus*). Each pond was stocked with juveniles of 0.3 g initial weight at a density of 200 snails m<sup>-2</sup>. This study provides good results in growth and production of spotted babylon in earthen ponds. The average growth rates in body weight were 0.67 and 0.51 g mo<sup>-1</sup> for snails held in the monoculture and polyculture with sea bass, respectively. At the end of the experiment, total yield of spotted babylon held in the monoculture and polyculture with sea bass was 10,520 and 10,450 kg ha<sup>-1</sup>, respectively. Based on farm data, stocking data and harvest data used in this study, total cost per production cycle of the monoculture and polyculture with sea bass were \$19,184 and \$23,245, respectively. The cost of producing spotted babylon marketable sizes in this grow-out farm design was \$5.69 per kg and \$6.95 per kg for the monoculture and polyculture trials, respectively. At a farm gate price in 2003 of \$9.00 per kg resulted in a net return of \$11,124 and \$14,691 for the monoculture and polyculture with sea bass, respectively. The present study indicated the technically feasible, and economically attractive for monoculture and polyculture of *B. areolata* to marketable sizes in earthen ponds.

**KEY WORDS:** *Babylonia areolata*, *Lateolabrax japonicus*, monoculture, polyculture, earthen ponds, production, economic analysis

### INTRODUCTION

Recently, there has been considerable interest in the commercial culture of spotted babylon, *Babylonia areolata*, in Thailand resulting from a growing demand and an expanding domestic market of seafood, and a catastrophic decline in natural spotted babylon populations in the Gulf of Thailand. From an aquaculture point of view, the spotted babylon had many biological attributes, production and market characteristics necessary for a profitable aquaculture venture and it was considered a promising new candidate of aquaculture species for the land-based aquaculture industry in Thailand (Chaitanawisuti & Kritsanapuntu 1999). At present, the successful culture of spotted babylon juveniles to marketable sizes was operated in large-scale production using the flow-through seawater system in concrete/canvas ponds. However, this culture technique had many considerations in disadvantages of the culture purposes. Basically, it needed the high investment of pond construction, buildings and facilities, large area for pond construction, and operational costs, but the production and low economic returns is not high enough for commercial operations (Chaitanawisuti et al. 2002a, 2002b). Because many marine shrimp ponds (*Penaeus monodon*) have been abandoned or rested because of diseases, poor management and environmental degradation for a long time in Thailand, this study was then focused on the potential and feasibility for a pilot growing-out of the spotted babylon juveniles to marketable sizes in earthen ponds. This study may provide an opportunity to develop a sustainable aquaculture system for growing out of spotted babylon juveniles to marketable sizes in earthen ponds and may result in the best use of many abandoned/rested shrimp ponds in coastal areas of Thailand. However, lack of economic data can be an important constraint to the

successful development of spotted babylon aquaculture operations. A financial investment analysis which tied biological, production, cost and market price variables has been used to make decisions about culture methods, feasibility and potential for commercial operation of this enterprise. However, polyculture techniques have been used to increase production of fish and shellfish in culture ponds. Several marine shellfish species have been shown in polycultures, augmenting harvests through wider use of available food and space, whereas minimizing the negative effects of species—specific competitions and exometabolites. In addition, the polyculture of shellfish with fish presented some possibility that could benefit the local aquaculture operation (Hunt et al. 1995). A lack of economic data can be an important constraint to the successful development of spotted babylon aquaculture operations. A financial investment analysis which tied biological, production, cost and market price variables has been used to make decisions about culture methods, and feasibility and potential for commercial operation of this enterprise. Thereafter, the land-based aquaculture operation for growing-out of spotted babylon in earthen ponds was developed for commercial purposes in Thailand. The objective of this study is to present the growth, production and economic consideration for monoculture of juvenile *B. areolata*, and their polyculture with sea bass, *L. calcarifer*, using large-scale production of earthen ponds.

### METHODS

#### Pond Design and Construction

This study was conducted at the Research and Development Unit for Aquaculture of the spotted babylon, Aquatic Resources Research Institute, Chulalongkorn University, Petchaburi province, Thailand, during 2003 to 2004. A total farm area of 0.8 ha was comprised of 0.32 ha grow-out earthen ponds, 0.4 ha seawater

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reservoir and 0.08 ha accommodation and office. Eight  $20.0 \times 20.0$  m earthen ponds and 1.5 m in depth were used for the polyculture trials. Ponds were arranged in a  $4 \times 4$  array with common walls to reduce construction costs and pond wall was 1.5 m in height, 3.0 m in width at the base and 2.5 m in width at the top. Ponds bottom was covered with coarse sand of approximately 10–15 cm in thickness. Each grow-out pond was fenced by plastic net of 15.0 mm mesh size and 1.2 m in width, supported with bamboo frame for strengthening. The plastic net must be buried under sand about 6 cm in depth to limit movement of snails along pond bottom and pond wall, and ease for harvesting. Prior to the start of the grow-out, all ponds were dried for 2 wk, and filled with ambient, unfiltered natural seawater from a nearby canal to a depth of 70 cm. Water level in the ponds was maintained at 70 cm by adding seawater to replace water loss caused by seepage and evaporation. The grow-out ponds are supplied with ambient unfiltered, natural seawater from seawater intake system. The seawater system was powered by one 5.5-hp engine equipped with water pump of 12.5 cm in diameter of outlet pipe. The seawater intake consisted of a 12.5 cm in diameter PVC pipe manifold horizontally into the sea. Seawater is delivered to each pond through main unlined canal of 80-cm width and 30-cm depth, and 15.0-cm diameter PVC distribution pipes (inlet). The drainage pipe of 12.5 cm in diameter PVC pipe was used as an outlet. Two air blowers (2 Hp) were used to supply high volume of air for all grow-out ponds. PVC pipes of 2.54 cm in diameter were connected to the outlet of the air blower and extended to the pond dike of each pond. Four polyethylene pipes of 18 m long and 1.6 cm in diameter was connected to the PVC pipe and extended to the bottom of each pond. On the PE pipe, there were 10 holes of 1.5 mm in diameter, and the distance between adjacent holes was 2 m. The PE pipes were sustained at 10 cm off the pond bottom using bamboo sticks. An aerator was operating daily for 16–20 h except during feeding and resting of blower.

#### *Monoculture and Polyculture Trials*

Spotted babylon and sea bass juveniles were purchased from private hatchery. Individuals from the same cohort were sorted by size to minimize differences in shell length (maximum anterior-posterior distance) and prevent possible growth retardation of small babylon when cultured with larger individuals. The spotted babylon juveniles had an average shell length and body weight of 1.0 cm and 0.3 g, respectively, and 12.7 cm and 37.2 g for those of sea bass, respectively. Two treatments of monoculture and polyculture were designed as following:

##### *Treatment 1: Monoculture of Spotted Babylon*

Initial stocking density of spotted babylon juveniles was 200 individuals  $\text{m}^{-2}$  (80,000 snails per pond).

##### *Treatment 2: Polyculture of Spotted Babylon with Sea Bass*

Initial stocking density of spotted Babylon and sea bass juveniles were 200 individuals  $\text{m}^{-2}$  (80,000 snails per pond), and 5 fish  $\text{m}^{-2}$  (2,000 fish per pond), respectively.

#### *Grow-out Operation*

The feeding schedule was as follows: sea bass were fed to satiation with fresh trash fish twice daily in morning (09:00 h) and evening (17:00 h). Spotted babylon were fed with fresh trash fish at 15% to 20% of body weight once daily in morning (09:00 h) this

was done after stopping feeding of the sea bass. Feeding was monitored daily by means of baited traps. Food amounts were adjusted every 30 d after body weight measurement. Fifty percent of seawater was exchanged at 15 d interval and seawater was sampled before water exchange at 25 cm above pond bottom for analyses of seawater temperature, salinity, pH, alkalinity, nitrite-nitrogen and ammonia-nitrogen following standard methods as described by APHA et al., 1985. Dissolved oxygen was measured daily. No chemical or antibiotic agent was used throughout the entire experimental periods. Grading by size was not carried out in any pond throughout the growing-out period. For growth estimation, 50 baited traps were used to sample the spotted babylon in each pond at 30 d interval for measurement of body weight individually and counting the number of snails per kg. The spotted babylon juveniles were cultured until they reached the marketable size of 120–150 snails per  $\text{kg}^{-1}$ .

#### *Economic Evaluation*

The components of financial analysis were categorized according to initial investment, annual ownership costs and annual operating costs as follows:

Initial investment requirements for farm construction on monoculture of juvenile spotted babylon and polyculture with sea bass to marketable sizes in earthen ponds were evaluated. The investment requirements included land, construction of eight  $20.0 \times 20.0 \times 1.5$  m grow-out earthen ponds, one 0.4 ha seawater reservoir, two seawater pumps and housing, two blowers and housing, four  $3.0 \times 5.0 \times 0.7$  m canvas nursery tanks and housing, accommodation for labor and office and operating equipment and facilities.

Ownership costs per production cycle consisted of land, depreciation and interest on investment. These costs are fixed and incurred in the short run regardless of whether the facilities are in operation. Annual depreciation was estimated by the straight-line method, based on the expected useful life of each item of equipment. Assets are assumed to have no residual value for all items constituting facilities at the end of their useful life. Eight  $20.0 \times 20.0 \times 1.5$  m grow-out earthen ponds and one 0.4 ha seawater reservoir were assumed to have useful life of 5 and 2 y, respectively. Housing and the blowers and seawater pumps were assigned a useful life of 2 y. The life expectancies of equipment ranged from 1–2 y. Interest rates for capital cost were based on 2003 bank loan rates (3.5% per year) for this type of business enterprise.

Operating costs per production cycle are incurred upon actual operation of the grow-out unit and include repairs and maintenance, labor, feed, utilities and interest on operating capital. Costs for purchasing and transportation of spotted babylon and sea bass juveniles are \$0.02 and 0.11 per juvenile, respectively. Spotted babylon and sea bass are fed fresh meat of trash fish at a cost of \$0.13 per 0.18/kg and feed conversion ratio was 2.2. The repairs and maintenance is estimated on the actual expenses for housing, earthen ponds, reservoirs, and operating equipment costs. Electricity is used for operating the various pumps and lighting units in the farm. The average charge was \$0.03 per kilowatt hour. Labor requirements were based on the particular needs for production cycle of the proposed farm. Two operators (full-time) were assigned for operation of the farm; the cost of one operator was \$125.0 per month. Operating equipment (fuel, storage containers, farm equipment, etc.), chemicals and lime was estimated based on

actual use of each item. Land is actual lease from private sector at a rate of \$625 per ha per year. Interest charges for operating capital are based on 2003 bank loan rates (3.5% per year) for this type of business.

#### Return Analysis

Net return and return on investment for grow-out production were computed at the selling price of spotted babylon market sizes at farm gate in 2003 ranging from \$8.8–9.3/kg. Gross return was computed from total yield multiplied by selling price. Net return was calculated from the gross return minus to the total amount cost per production cycle. (Rubino 1992, Fuller et al. 1992).

### RESULTS

#### Growth and Production

Growth expressed as body weight and number of snail per kilogram of juvenile *B. areolata* for monoculture and polyculture with sea bass over a period of 7 mo was shown in Figure 1 and Figure 2. Growth of spotted babylon was not significantly higher in monoculture, compared with growth in polyculture with sea bass ( $P < 0.05$ ). The average growth rates in body weight were 0.67 and 0.51 g mo<sup>-1</sup> for snails held in the monoculture and polyculture with sea bass, respectively. Mean ( $\pm$ SE) final body weights of spotted babylon held in the monoculture and polyculture with sea bass was 5.22  $\pm$  0.63 g, and 4.10  $\pm$  0.57 g, respectively. Mean ( $\pm$ SE) final shell lengths of snails held in the monoculture and polyculture with sea bass was 3.2  $\pm$  0.35 cm, and 3.6  $\pm$  0.75 cm, respectively. The snails can reach the sizes of 205  $\pm$  17.55 and 214  $\pm$  43.90 individuals kg<sup>-1</sup> for the monoculture, polyculture with sea bass, respectively. Feed conversion ratio (FCR) was 2.69 and 2.71 for snails held in the monoculture and polyculture with sea bass, respectively. Final survival of snails held in the monoculture was 84.94%, and 84.30% for those held in polyculture with sea bass, respectively. At the end of the experiment, the average total yield of spotted babylon in monoculture and polyculture with sea bass were 10,525 and 10,450 kg ha<sup>-1</sup>, respectively. Size distribution of the spotted babylon in monoculture was consisted of 2 main size classes of 100–250 and <250 snails /kg with 90.81% and 9.19%, respectively. For Polyculture with sea bass, the snails consisted of 2 main size classes of 100–250 and <250 snails kg<sup>-1</sup> with 87.57% and 12.43%, respectively. For sea bass, the average final weight, final survival, FCR and total production were 300–1,200 g, 46.0%, 4.47 and 12,250 kg ha<sup>-1</sup>, respectively.

#### Economic Evaluation

Farm data (total farm area, pond sizes and total pond area), stocking data (average initial weight, stocking density) and harvest data (duration of grow-out, average weight at harvest, final survival, feed conversion ratio and yield) are based on the actual data of pilot farm. Parameters used for the economic analysis for monoculture and polyculture of spotted Babylon with sea bass in a total farm area of 0.8 ha were summarized in Tables 1, 2, 3, 4, 5. Total investment requirement for construction of a total farm area of 0.8 ha was estimated to be \$4,837 for the monoculture and polyculture trials. Construction of grow-out ponds and seawater reservoirs was the largest cost component of the farm (35.14% of the total investment cost), followed by building of canvas nursery ponds, land, seawater pumps and blowers representing 12.92%, 10.34%, 10.34% and 10.34% of the total investment cost, respec-

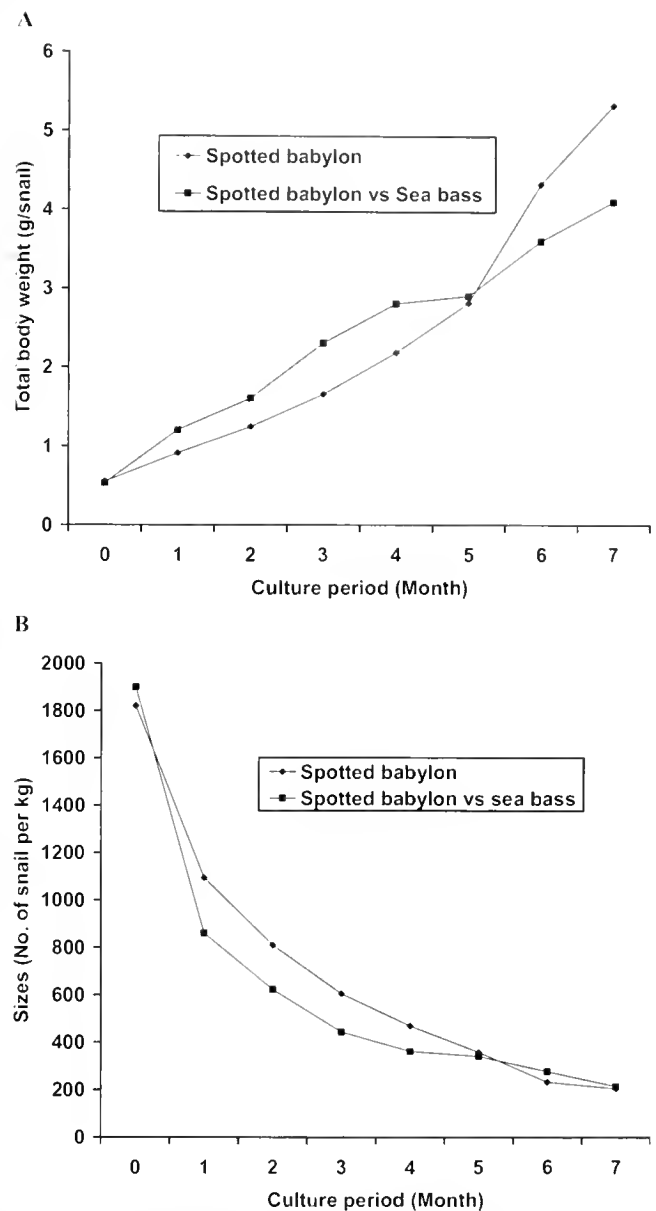


Figure 1. (A) Growth in body weight of juvenile *B. areolata* held in monoculture and polyculture with sea bass in earthen pond. (B) Growth in sizes (number of snails per kg) of juvenile *B. areolata* held in monoculture and polyculture with sea bass in earthen pond.

tively (Table 2). Ownership cost per production cycle was estimated to be \$2,241 for the monoculture and polyculture trials. The major ownership cost items were depreciation, land and interest on investment accounting 76.22%, 22.31% and 1.47% of total ownership cost, respectively (Table 3). Operating costs per production cycle was estimated to be \$16,943 and \$21,004 for the monoculture and polyculture trials, respectively (Table 4). Total cost per production cycle for the monoculture of spotted babylon in a total farm area of 0.8 ha was \$19,184 and \$23,245 for the monoculture and polyculture trials, respectively (Table 5). The cost of producing spotted babylon marketable sizes in this grow-out farm design was \$5.69 per kg and \$6.95 per kg for the monoculture and polyculture trials, respectively. The enterprise budgets based on the price of spotted babylon at farm gate in 2003 of \$9.00 per kg

TABLE 1.

Parameters used for the economic analysis for the monoculture and polyculture trials of spotted babylon in a total farm area of 0.8 ha of earthen ponds.

Parameters	Monoculture	Polyculture
	Amount (\$)	Amount (\$)
A. Farm data		
Total farm area (ha)	0.8	0.8
Pond size (ha)	0.04	0.04
Total pond area (ha)	0.3	0.3
Total area of seawater reservoirs (ha)	0.4	0.4
B. Stocking data		
Average initial weight of spotted babylon (g)	0.3	0.3
Average initial weight of sea bass (g)		2.3
Stocking density of spotted babylon (no. m <sup>-2</sup> )	200	200
Stocking density of sea bass (no. m <sup>-2</sup> )		5
C. Harvest data (Spotted Babylon)		
Duration of grow-out (mo)	7	7
Average number of crops per year per pond	1.4	1.4
Average final weight (g)	5.22	4.10
Average final survival (%)	84.94	84.30
Feed conversion ratio (FCR)	2.69	2.71
Yield per production cycle (kg/ha)	10,520	10,450
Selling price at farm gate (\$/kg)	8.75–9.25	8.75–9.25
D. Harvest data (Sea bass)		
Duration of grow-out (mo)		6
Average number of crops per year per pond		2
Average final weight (g)		300–1,200
Average final survival (%)		46.00
Feed conversion ratio (FCR)		4.47
Yield per production cycle (kg/ha)		12,250
Selling price at farm gate (\$/kg)		1.89–2.25

resulted in net return of \$11,124 and \$14,691 for the monoculture and polyculture trials, respectively (Table 6).

### DISCUSSION

In this study, the average monthly growth rate, FCR and final survival of spotted babylon for monoculture in earthen pond were 0.67 g mo<sup>-1</sup>, 2.69, and 84.94%, respectively, and the average monthly growth rate, FCR and final survival of spotted babylon were 0.51 g mo<sup>-1</sup>, 2.71, and 84.30% respectively for polyculture with sea bass. The snails can reach a marketable size with an average body weight of 9–10 g in a 7-mo period of culture. By contrast, Chaitanawisuti and Kritsanapuntu (1999) reported that average monthly growth rates of spotted babylon in flow through culture system in concrete/canvas tanks was 1.4 g mo<sup>-1</sup>. FCR and final survival were 1.6% and 95.8%, respectively. Chaitanawisuti et al. (2001) reported polyculture of *B. areolata* and *L. calcarifer* in 3.0 × 4.5 × 0.5 m concrete ponds supplied with flow-through seawater system that the average growth, survival, FCR and total production were 1.17 g mo<sup>-1</sup>, 96.0%, 1.34 and 29.0

TABLE 2.

Estimated investments requirements for the monoculture and polyculture trials of spotted babylon in a total farm area of 0.8 ha of earthen ponds.

Items	Amount (\$)	%
Land renting	500	10.34
Construction of eight 20.0 × 20.0 × 1.5 m grow-out earthen ponds and one 0.4 ha seawater reservoirs	1,700	35.14
Construction of accommodation and storage house	250	5.17
Construction of four 3.0 × 5.0 × 0.7 m canvass nursery ponds and housing	625	12.92
Water pumps and housing	500	10.34
Blowers and housing	500	10.34
Traps for sampling and harvesting	100	2.06
Operating equipment (pvc pipes, lighting, salinometer, thermometer, ect)	162	3.35
Miscellaneous	500	10.34
Total investment	4,837	100

kg, respectively. Growth of spotted babylon in earthen ponds was lower than those in concrete/canvas tank. The most concerned major issues for slow growth of spotted babylon in earthen ponds is the soil sanitization caused by pond seepage, salinity increases caused by water evaporation, salinity decrease caused by heavy rain falls, fast deterioration of total alkalinity, appropriate feeding strategy and invasions of snails (*Cerithium* sp.) as follows: (1) excessive food caused the degradation of water quality and decay of pond bottom; (2) food competition from various predators such as the tiger prawn naturally occur in earthen ponds (*Peneaus monodon*), swimming crabs (*Portunus pelagicus*), mud crab (*Scylla* sp), carp (*Oreochromis mossambica*); (3) deterioration of water quality, particularly total alkalinity which, caused slower feeding of spotted Babylon; (4) salinity decrease during rainy season, which caused slower feeding and obvious slow growth and (5)

TABLE 3.

Estimated ownership costs per production cycle for the monoculture and polyculture trials of spotted babylon in a total farm area of 0.8 ha of earthen ponds.

Items	Amount (\$)	%
Land	500	22.31
Depreciation		
Construction of grow-out earthen ponds and seawater reservoirs	340	15.17
Construction of accommodations and facilities	125	5.58
Construction of canvass nursery ponds and housing	312	13.92
Water pumps and housing	250	11.16
Blowers and housing	250	11.16
Traps for sampling and harvesting	1,000	4.46
Equipment (pvc pipes, lighting, salinometer, thermometer, ect)	81	3.61
Miscellaneous	251	11.16
Interest on fixed cost	33	1.47
Total ownership cost	2,241	100

TABLE 4.

Estimated operating costs per production cycle for the monoculture and polyculture trials of spotted babylon in a total farm area of 0.8 ha of earthen ponds.

Items	Monoculture		Polycultures	
	Amount (\$)	%	Amount (\$)	%
Purchasing for juveniles spotted Babylon	11,200	66.10	11,200	58.32
Purchasing for juveniles sea bass	—	—	1,800	8.57
Fuels and lubricants	586	3.46	586	2.79
Electricity	378	2.23	378	1.80
Feed for spotted babylon	1,358	8.02	1,358	6.47
Feed for sea bass	—	—	1,920	9.14
Labor (2 full time)	1,750	10.33	1,750	8.33
Repairs and maintenance	375	2.21	375	1.79
Ice for feed storage	108	0.64	108	0.51
Interests on operating capital	1,188	7.01	1,529	7.28
Total operating cost	16,943	100	21,003	100

mineral competition from a large number of snails (*Cerithium* sp.) that competed for minerals in the seawater, particularly calcium for shell formation, which resulted in shell abnormality and slow growth. In this study, production and economic analysis was performed for monoculture of juvenile *B. areolata* to marketable sizes using a large-scale production of earthen ponds in Thailand. The analysis was based on actual cost and production data from a pilot commercial-scale farm. A total farm area of 0.8 ha was comprised of 0.3 ha grow-out earthen ponds, 0.4 ha seawater reservoir and 0.08 ha accommodation and office.

The enterprise budgets of monoculture based on the price of spotted babylon at farm gate in 2003 of \$9.00/kg, net return of the monoculture and polyculture were \$11,124 and \$14,691, respec-

TABLE 5.

Estimated total cost (%) per production cycle for the monoculture and polyculture trials of spotted babylon in a total farm area of 0.8 ha of earthen ponds.

Items	Monoculture		Polyculture	
	Amount (\$)	%	Amount (\$)	%
Ownership costs	2,241	11.68	2,241	9.64
Land	500	2.61	500	2.15
Depreciation	1,708	8.90	1,708	7.35
Interest on investment	33	0.17	33	0.14
Operating costs	16,943	88.32	21,004	90.36
Spotted Babylon juveniles	11,200	58.38	11,200	48.18
Sea bass juveniles	—	—	1,800	7.74
Fuel and lubricants	586	3.05	586	2.52
Electricity	378	1.97	378	1.63
Feed for spotted babylon	1,358	7.08	1,358	5.84
Feed for sea bass	—	—	1,920	8.26
Hired labor	1,750	9.12	1,750	7.53
Repairs and maintenance	375	1.95	375	1.61
Ice for storage of feed	108	0.56	108	0.47
Interests on investment	1,188	6.19	1,529	6.58
Total cost per production cycle	19,184	100	23,245	100

TABLE 6.

Enterprise budgets of a total farm area of 8,000 m<sup>2</sup> for the monoculture and polyculture trials of spotted babylon in a total farm area of 0.8 ha of earthen ponds.

Parameters	Monoculture	Polyculture
Production		
Spotted Babylon (kg)	3,368	3,344
Sea bass (kg)	—	3,920
Costs per production cycle		
Initial investment requirements	4,837	4,837
Ownership costs (\$)	2,241	2,241
Operating costs (\$)	16,943	21,004
Total cost (\$)	19,184	23,245
Returns		
Gross return (\$)	30,312	37,936
Net returns (\$)	11,124	14,691

\* Total yield of spotted Babylon and sea bass per production cycle at 0.4 ha

— Price at farm gate for spotted Babylon and sea bass of \$9.00 and 2.25/kg, respectively

tively. This study presented a positive net return and a payback period of less than five years are often used as business investment criteria. In Thailand, living spotted babylon fetched the selling prices ranging from \$11.25–15.00/kg at seafood restaurants and \$8.75–9.25 per kg at a farm outlets. The basic consumption in this study (juvenile price of \$0.02 per juvenile, production feed price of \$0.2 per kg, stocking density of 200 snails m<sup>-2</sup>, and selling price of \$9.0 per kg) indicated that the proposed eight 20.0 × 20.0 × 1.5 m grow-out earthen ponds operation is economically feasible under these conditions. The feasibility of producing spotted babylon marketable sizes in pilot commercial grow-out earthen pond operation should be continued to be examined. Although returns are small, production with 80% survival and selling price of \$9.0 per kg is economically feasible under the assumptions used. The results showed that total yield of monoculture (10,520 kg/ha) and polyculture with sea bass (10,450 kg/ha) was gradually different. For cost and returns analysis, total cost per production cycle of polyculture with sea bass (\$23,245) was 17.47% higher than that of monoculture (\$19,184) because of increasing costs of sea bass juveniles and feed, and the net return per production cycle of polyculture with sea bass (\$14,691) was 24.28% higher than the monoculture (\$11,124). Results of this work showed that juvenile spotted Babylon could be successfully grown to marketable size in earthen ponds for monoculture and polyculture systems.

This study has basically demonstrated that it is possible to culture the spotted Babylon in earthen ponds such as the abandoned/rested shrimp ponds by stocking acclimated spotted babylon juveniles to marketable sizes. Thus, monoculture and polyculture of spotted babylon is environmentally friendly and economically attractive with appropriate abandoned/rested shrimp farms, resulting in effective reuse of abandoned shrimp ponds, better economic returns and less environmental pollution. Furthermore, the polyculture of spotted babylon with sea bass or milkfish at relatively low stocking density may provide an opportunity to develop a sustainable aquaculture system to best use many abandoned/rested shrimp ponds in various coastal areas of Thailand. The results of this study provide preliminary evidence for the biological feasibility of culturing the spotted Babylon, *B. areolata*, in earthen ponds for monoculture and polyculture. However, application of

these results to commercial levels of production should be preceded by careful examination of other parameters that might be important, such as deterioration of water quality at high stocking densities. Further study should concentrate on pond design, management of seawater and pond bottom quality, feeding strategy and competition for food and habitat caused by natural occurrence of organisms, for the success of commercial grow-out operation of spotted babylon in earthen ponds.

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## THE INVASIVE RAPA WHELK *RAPANA VENOSA* (VALENCIENNES 1846): STATUS AND POTENTIAL ECOLOGICAL IMPACTS IN THE RÍO DE LA PLATA ESTUARY, ARGENTINA-URUGUAY

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**ABSTRACT** Recent range extensions of the invasive rapa whelk *Rapana venosa*, biological data of the population and possible ecological impacts on the food webs of the Río de la Plata estuary are presented. A total of 41 rapa whelks and 21 egg capsules were collected between February 2004 and March 2006. Specimens were found all over the mixohaline waters of the estuary, with records off Montevideo, Samborombón Bay and off Punta Rasa. Specimens ranged between 28 and 120 mm shell length. Almost all *R. venosa* presented epibionts all over the shell, suggesting an exposed lifestyle. Egg-masses were attached to specimens of *R. venosa*, debris and plastic garbage. The spatial distribution of *R. venosa* within the estuary was coupled with the spatial distribution of *Macra isabelleana*, a typical subtidal bivalve of mixohaline waters. The implications for the food webs of the estuary, including possible predators, are discussed. The presence of *R. venosa* in muddy sediments together with the finding of egg-capsules over the specimens, and the low salinity values at which it is found in the Río de la Plata estuary, confirmed the high plasticity of *R. venosa* and the potential capability of successfully invading novel environments.

**KEY WORDS:** exotic species, invasion, *Rapana venosa*, Muricidae, Rapa whelk, *Macra isabelleana*, *Ostrea puelchana*, food webs, Río de la Plata, Southwestern Atlantic

### INTRODUCTION

The large Asian gastropod *Rapana venosa* Valenciennes 1846 (Neogastropoda, Muricidae) is a predatory mollusc native to the Sea of Japan, Yellow Sea, Bohai Sea and the East China Sea to Taiwan (ICES, 2004). This species was discovered out of its native biogeographic range in the Black Sea, and it has subsequently spread throughout the Sea of Azov, and the Aegean, Adriatic and North Seas (for complete lists of localities see ICES, 2004). The first collection of *R. venosa* in North America was made in the Chesapeake Bay on the East Coast of the United States in 1998 (Harding & Mann 1999). The first report of rapa whelks in South America was made by Scarabino et al. (1999) and Pastorino et al. (2000) from collections in the Río de la Plata estuary, Argentina-Uruguay.

This generalist predator of subtidal mollusks usually feeds on bivalves of economic interest like oysters, mussels and clams (Harding & Mann 1999, Savini et al. 2004), and has been identified as the prime reason for the collapse of several banks of mussels and oysters in the Black Sea (Drapkin 1963, Zolotarev 1996). Adult *Rapana venosa* are found in water temperatures between 4°C and 27°C (Chung et al. 1993) and salinities ranging from 28–33 in Korean waters (Korea Oceanographic Data Center, <http://kode2.nfrdi.re.kr:8001/home/eng/main/index.php>). Furthermore, in nonnative localities *R. venosa* is found in estuarine salinities (Scarabino et al. 1999, Pastorino et al. 2000, Mann & Harding 2000, 2003; ICES 2004). Their high fecundity (Chung et al. 1993), dispersal assisted by a planktonic larvae that is capable of remaining planktonic between 14 and 80 days in estuarine and marine waters (Mann & Harding 2003), and fast growth (Harding & Mann

2005) make *R. venosa* a potentially successful invader worldwide (Savini et al. 2004).

A recent general review of exotic species in the Southwestern Atlantic (34°S to 54°S) revealed that most of the exotic species of the region are concentrated in Buenos Aires Province (northern Argentina), where major commercial harbors are located (Orensanz et al. 2002). At this region, the Río de la Plata (34° to 36°30'S, 55° to 58°30'W) forms one of the largest estuarine systems of South America (ca. 38,000 km<sup>2</sup> of mixohaline area). This river is one of the large waterways of South America. Two of the major ports in the region, Buenos Aires (Argentina) and Montevideo (Uruguay), lie along its shores generating an intense nautical traffic. The estuary is also the maritime access to the highly complex fluvial system named "Hidrovia," communicating with the Amazon Basin (Bisbal 1995, Mianzan et al. 2001).

Although it is known that ships are one of the main sources of introduction of exotic species via fouling or ballast water, regulations regarding the later in Argentina are not strictly enforced (Orensanz et al. 2002). In agreement, this was the mechanism suggested for the introduction of *Rapana venosa* in the Río de la Plata estuary (Pastorino et al. 2000). Early collections of *Rapana venosa* in the estuary corresponded to adult specimens found in the Rouen and English Banks in May 1998, several egg masses along the Uruguayan coast, between Montevideo and Punta del Este in December 1998 to February 1999 (Scarabino et al. 1999), one specimen (95 mm shell length) in November 1999 off Samborombón Bay (Pastorino et al. 2000), another specimen (89 mm shell length) collected in November 2001 off Montevideo (Rodríguez Capitulo et al. 2002), and recently (spring 2002), several specimens caught in a fisheries research survey along the outer part of the estuary (Carranza et al. in press) (see Fig. 1). However, studies about the potential extent of establishment of this gastropod over time or possible ecological impacts on the food webs of the estuary

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haline waters of the estuary, with constant records off Montevideo and in Samborombón Bay. In 2005 and 2006 *R. venosa* was found in southern locations, at higher salinities off Punta Rasa (see Fig. 2 and Table 2). Specimens ranged between 28 and 120 mm SL. SL-frequency and distribution of *Rapana venosa* in the estuary for each cruise is shown in Figure 2. Total weight ranged between 2.8 g and 351.5 g. Figure 3 shows the result of the regression analysis between SL (independent variable) and TW (dependent variable).

Rapa whelks were collected in shallow muddy (36 specimens) and sandy (5 specimens) bottoms. The associated fauna in muddy bottoms were the bivalve *Macra isabellana* (see Fig. 2d), the shrimp *Artemesia longinaris*, the gastropods *Buccinanops* sp. and *Heleobia australis*, the polychaete *Alitta succinea* and the cumacean *Diastylis* sp. In sandy bottoms *R. venosa* was found together with the oyster *O. puelchana* in three sampling stations (see Fig. 2d for the distribution of possible preys).

Undetermined bryozoans (63%) and barnacles (*Balanus* sp., 78%) were the most frequent epibionts colonizing all over the shell of *Rapana venosa* ( $n = 36$ ). Hydrozoan branches (30%) were also

found. Undetermined small sea anemones, undetermined chitons and polychaete tubes (Serpulidae) were found occasionally. Five rapa whelks lack epibionts completely. *Polydora* sp. (Polychaeta) infestations were found in 6 specimens.

A total of 21 egg masses were found. Eight of them were attached to debris and plastic garbage, whereas 13 were found attached to larger whelks. Capsules ( $n = 30$ ) per whole egg mass varied between 108 and 700, and eggs per capsules varied from 434–890. Fecundity estimations were in the range 76,156–85,323 eggs per individual. The egg capsules contained shelled larvae having the operculum and larval shell ( $\sim 0.4 \times 0.3$  mm). Larvae with “coned” shells were found with a frequency of  $\sim 10\%$  (Fig. 4). Egg capsules from December were pale black color (average of 717 eggs per capsule), whereas several capsules from February were empty with the exit hole bored, or dead with a violet color (average of 568 eggs per capsule), and capsules from March were all empty. Collected capsules, including the curved tip, measured 18.5–29.5 mm in length, and 1.7–2.5 mm in width at the smaller diameter at the base.

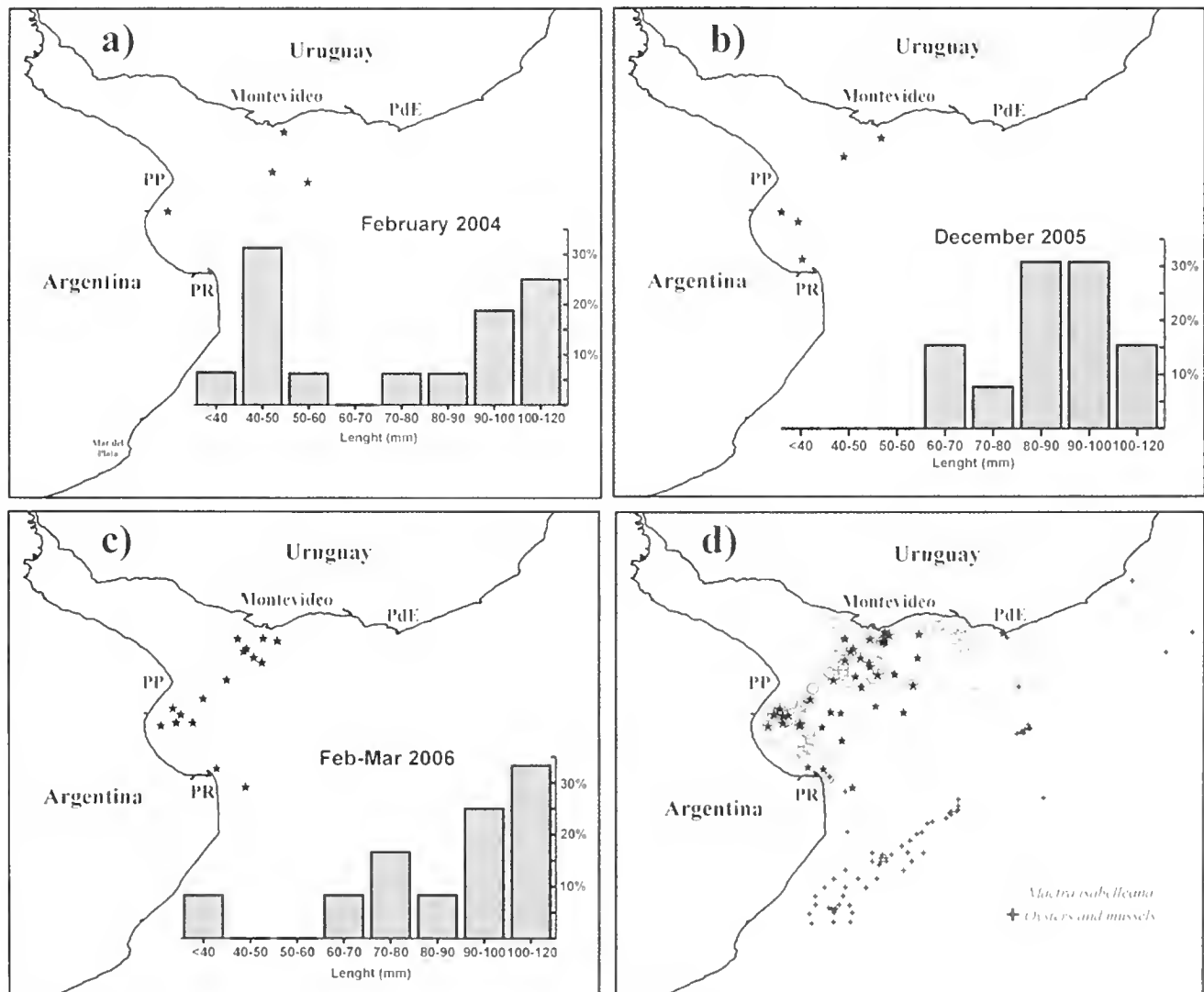


Figure 2. Shell length-frequency and distribution of *Rapana venosa* (★) in the study area during each cruise (a to c). Complete known range distribution of *R. venosa* (★) and potential bivalve preys (banks of *Macra isabellana*, *Ostrea puelchana* and *Mytilus edulis platensis*) (d). PP, Punta piedras; PR, Punta Rasa; PdE, Punta del Este.

TABLE 2.  
Collection of *Rapana venosa* and biometric variables in the Río de la Plata estuary.

Sampling Date	n	Shell Length (mm)	Aperture Height (mm)	Aperture Width (mm)	Thickness (mm)
February 2004	16	36.82–111.92	28.4–94.9	11.3–44.8	0.6–4.9
December 2005	13	68.8–106.8	56–89.2	24.3–41.7	1.6–3.3
Feb–March 2006	12	28.6–119.3	21.5–99.1	9.04–52.5	0.5–3.4

## DISCUSSION

The rapa whelk *Rapana venosa* successfully spread over the muddy bottoms of the estuary since their early records off Samborombón Bay and along the Uruguayan coast (Pastorino et al. 2000, Scarabino et al. 1999, Rodríguez Capitulo et al. 2002). Their range extension is now at Argentinean and Uruguayan coastal lines. The finding of invasive marine species in coastal and estuarine waters is an already widespread phenomenon. The impacts of such introductions are unpredictable, and not all of them become a successful resident in the new environments. However, the presence of small specimens of rapa whelk (between 28 and 48 mm SL), and egg capsules with operculated and shelled larvae, confirmed that the population of *R. venosa* is sexually mature and actively breeding in the region, as was suggested in previous works (Scarabino et al. 1999, Pastorino et al. 2000).

Biometric variables measured were in the range reported by Savini et al. (2004) for the Northern Adriatic Sea for similar shell length ranges. Besides their smaller size, the allometric growth of the snails collected in this study presented similar slope values than larger Adriatic species (3.21 and 3.37 for rock and sand samples, Savini et al. 2004). The record of small specimens in other localities where *R. venosa* was introduced is a rare event, because most of the data usually come from commercial fisheries that selectively catch larger snails (Harding & Mann 1999, ICES

2004) or because of possible distinct habitat preferences of juveniles (Savini et al. 2004). However, our data from the Río de la Plata estuary do not show segregation by size classes.

Almost all *Rapana venosa* specimens presented epibionts all over the shell, which suggests an exposed lifestyle. Similar results were found by Savini et al. (2004) for specimens living on hard rock. On the contrary, other studies under field and laboratory conditions found that rapa whelks are nocturnal and remain burrowed most of the day, avoiding settlement by epifaunal biota (Harding & Mann 1999, 2005). Environmental conditions in the Río de la Plata estuary may conduct to a different behavior. The light penetration in mixohaline waters is really poor, with approximately 99% of the incident radiation lost at around 2 m depth (Acha et al. submitted), and the sedimentation rate is high (Urien 1972), which means that adult rapa whelks may be exposed during longer periods of time than in other habitats. It is also remarkable that the organisms that encrust the shells of this gastropod are not usually found in the muddy bottoms of the estuary (Giberto et al. 2004), with the exception of barnacles colonizing small specimens of *Maetra isabelleana* (Giberto, pers. obs.). This reflects the lack of hard bottoms in the estuary and the importance of *R. venosa* shells as suitable settlement substrates for epibionts larvae. Moreover, *R. venosa* egg-masses were attached to debris and plastic garbage, which are concentrated over the Barra del Indio shoal by the frontal dynamics (Acha et al. 2003).

Habitat quality is very important for the successful colonization of novel environments. Salinity ranges of the estuary are within the limits at which the species has been found in other locations (ICES 2004), although in this area *Rapana venosa* was always found at low salinity waters (see Table 1 and Carranza et al. in press). Thermal seasonal range of the estuary (10°C to 23°C, Guerrero et al. 1997) is in coincidence with breeding tolerances for *R. venosa* (13°C to 26°C) recorded by Chung et al. (1993). These authors also reported a 17-day incubation period at 18°C to 20°C in laboratory conditions, with females laying egg-masses during all the reproductive period in summer. Specimens of this estuary seem to follow this pattern, because the egg-capsules found in November contained embryos at a morula-gastrula stage (Pastorino et al. 2000), whereas the capsules found in this study (December–February) were with normal and “coned” shelled larvae or empty. “Coned” shells could be malformations leading to nonviable larvae. Egg-masses were attached to specimens of *R. venosa* and debris and plastic garbage, which seems to be an alternative reproductive strategy to deal with the absence of any other primary settlement substrate. These findings are in coincidence with data from the outer estuary by Carranza et al. (in press), which also found egg masses attached to plastic debris and garbage.

The spatial distribution of *Rapana venosa* within the estuary

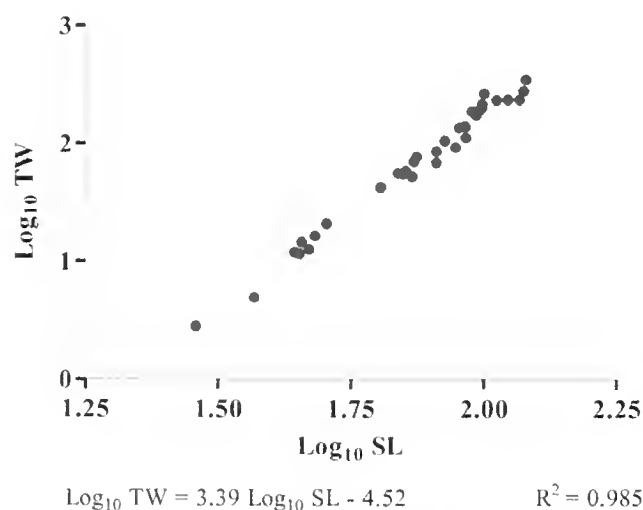


Figure 3. Linear relationship (data  $\log_{10}$  transformed) between shell length (mm) (independent variable) and total weight (g) (dependent variable) for samples of *Rapana venosa* collected in the Río de la Plata estuary between 2004 and 2006. Equation and regression coefficient ( $R^2$ ) are indicated. SL, shell length; TW, total weight.

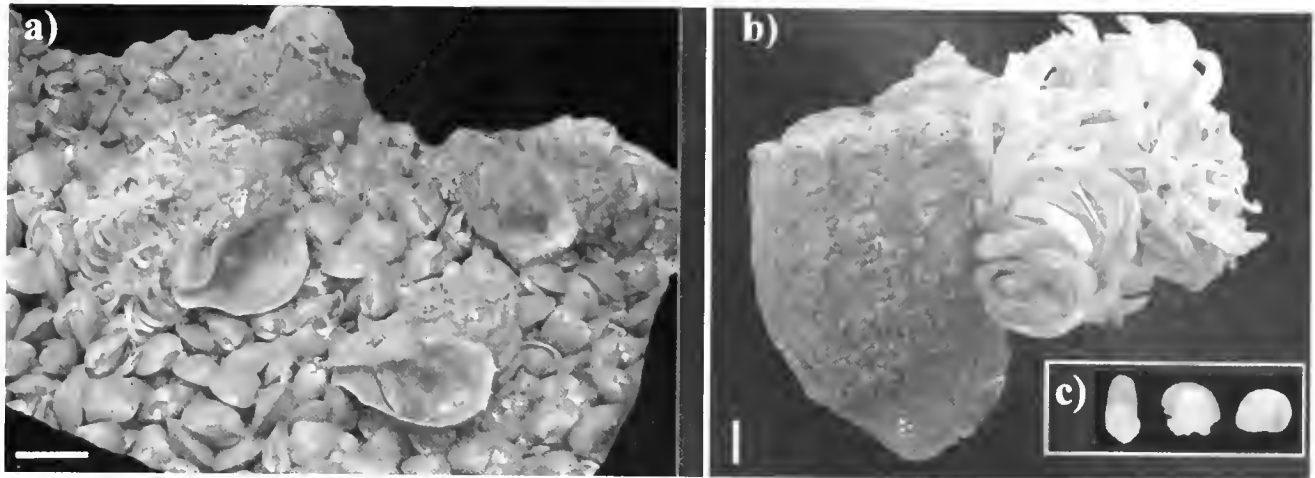


Figure 4. Adults of *Rapana venosa* collected in the Río de la Plata estuary over *Mactra isabelleana* banks (a, scale bar: 50 mm). Egg cases were attached to the specimens (b, scale bar: 10 mm) and contained "coned" shells forms and normal shelled larva of  $0.4 \times 0.3$  mm (c).

was coupled with the spatial distribution of *Mactra isabelleana*. This is in coincidence with the salinity range of 12–30 and the presence of muddy bottoms at which *M. isabelleana* is usually found at high densities (Giberto et al. 2004). It has been proposed that the high abundances of this deposit-feeder bivalve are related to the concentration of organic matter by frontal dynamics, and/or to the retentive properties of the front that could maintain bivalve larvae in this area (Mianzan et al. 2001, Giberto et al. 2004). At the adjacent subtidal marine zones, *R. venosa* was found near oyster (*Ostrea puelchana*) banks. The coexistence of *R. venosa* with these bivalves led to conjecture that it is preying over *M. isabelleana* and *O. puelchana*. Preliminary results on the trophic food webs in the estuary, using stable  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  isotope signatures (Botto et al. In prep.), supported this hypothesis: *R. venosa* ( $n = 4$ ) showed  $\delta^{15}\text{N}$  isotopic values in an upper trophic level, over both bivalves signals. Other potential preys could be the bivalves *Erodona macroides*, *Corbula patagonica* and *Nucula puelcha*, which usually inhabit the mixohaline environment (Giberto & Bremec 2003, In prep., Giberto et al. 2004).

Regarding possible predators, it has been suggested that *Callinectes sapidus* could be a control for *R. venosa* in the Chesapeake Bay (Harding 2003), but Cesar et al. (2003) has shown that this crab (which is another exotic species in this estuary) has a diet mainly based on *Limnoperna fortunei*. This is an introduced bivalve typical of low salinity to freshwaters of the Río de la Plata. Other possible natural controls are demersal fishes that prey on the infauna. The whitemouth croaker, *Micropogonias furnieri*, is the dominant species in terms of biomass and sustains the coastal and artisanal fisheries in Argentina and Uruguay (Carozza et al. 2004). *M. furnieri* usually preys on benthic infauna including soft-bodied polychaetes and hard-bodied crustaceans, gastropods and bivalves

like *M. isabelleana* (Puig 1986, Giberto 2001, Giberto unpublished data), using their pharyngeal teeth to crush the hard structures of these invertebrates. Therefore, *M. furnieri* is potentially capable of preying on *R. venosa* juveniles or at least on the egg-masses, the later confirmed recently in diets studies of this croaker (Giberto, unpublished data).

Considering the prolonged larval phase of *R. venosa* (up to 80 days), the lack of potential competitors in the estuary such as stable populations of large gastropods (Giberto et al. 2004, Carranza et al. in press, Giberto & Bremec In prep.), and the impact of the Río de la Plata discharge along the Uruguayan and Argentine coasts, the species is capable of maintaining a source population in the estuary. The vicinity of several other commercial harbors, in combination with favorable oceanographic conditions and food availability leads to the conclusion that viable populations of *R. venosa* are potentially expected in surroundings areas in a short term. The presence of *R. venosa* in muddy sediments together with the finding of egg-capsules over the specimens, and the low salinity values at which it is found in the Río de la Plata estuary, confirmed the high plasticity of *R. venosa* and the potential capability of successfully invading novel environments.

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## REPRODUCTIVE CYCLE OF THE PURPLE SNAIL *PLICOPURPURA PANSA* (GOULD 1853) FROM TWO LOCATIONS AT BAJA CALIFORNIA SUR, MEXICO

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**ABSTRACT** The reproductive cycle of purple snail, *Plicopurpura pansa* (Gould 1853), sampled randomly monthly over a period of 20 mo at Playa Cerritos at the Pacific, and Punta Perico at the Gulf of California, Baja California Sur, Mexico, was examined by histological observations of the gonadal development. At both sites year-round copulations were observed, whereas egg capsules could be found only during February to May. During nearly the whole period, male and female gonads were found in the developing stage. From January to July and August most female gonads were found in the ripe, and from January to June and August in the spawning stage. Male gonads in the spawning stage were observed from December to September. The recovering stage was found in male and female gonads between September and October. Synchronism of gonadal development between both sexes was evident. Spawning could be observed in females with a total shell length of more than 18 mm, whereas smaller animals had gonads in the developing stage. In gonads of males signs of spawning could be observed in animals larger than 18 mm. No relationship was found between the water temperature and the spawning period. The sex ratio was 1:1 at Playa Cerritos, whereas at Punta Perico more males than females were counted.

**KEY WORDS:** reproductive cycle, purple snail, *Plicopurpura pansa*, Muricidae

### INTRODUCTION

Most marine snails of the family Muricidae, if not all, produce in the hypobranchial gland a colorless secretion that turns on exposure to air and light to “Tyrian Purple” (Fretter & Graham 1994). For its dye the carnivorous, gonochoristic muricid *Plicopurpura pansa* (Gould 1853), which inhabits intertidal rocky shores exposed to high impact waves of the open sea, has been exploited on the Pacific coast from Central America to Mexico for hundreds of years by indigenous communities for dyeing threads of cotton to be woven afterwards into traditional dresses. However, in recent years in Mexico the commercial exploitation of the purple snail for dyeing kimonos with “Tyrian Purple” had reached such levels as to threaten the survival of the species, and in 1988 the Mexican government declared *P. pansa* a protected species (Anonymous 1988, 1994).

Despite the recent concerns about the state and recovery of *P. pansa*, little is known about the principal life-history features. The impeded accessibility, only during extreme low tides, is the main reason for the difficulties of *P. pansa* field research. Detailed information on the reproductive strategy however, is a prerequisite for the development of techniques for restocking natural populations and to facilitate effective management.

In Mexico, some information about the gonad reproductive cycle of the purple snail *P. pansa* is available from Oaxaca (Hernández-Cortés & Acevedo-García 1987, Acevedo-García et al. 1993), a histological study from Nayarit (Quiroz-Rocha 1992), and a detailed study about the reproductive cycle from Sinaloa (González-Flores 1997). Especially, the understanding of the reproductive cycle and the spawning period of *P. pansa* will provide the necessary information needed for the determination of the recruitment period of natural populations. Up until now data are missing about the reproductive cycle of *P. pansa* from Baja California Sur, the most northern area of its distribution. The objectives of this study were to determine over a period of 20 mo the reproductive cycle of *P. pansa* at two locations in Baja California

Sur. Additional information on minimum size at sexual maturity and sex ratio is needed to facilitate efficient management of this natural resource.

### MATERIALS AND METHODS

#### Sampling

Preliminary trials were started from January to May 2000 to determine the best location and period for the collection of *P. pansa*, whether egg capsules can be found, and to assess the method of sampling to obtain statistically valid data. From intertidal rocks at days during extreme low-water spring tides at Playa Cerritos at the Pacific coast (23°19'54"N and 110°10'38"W) and at Punta Perico at the Gulf of California (24°01'54"N and 109°48'21"W; Fig. 1), 20–30 specimens from 100–300 animals of unexploited populations of *P. pansa* were randomly selected from June 2000 to January 2002, monthly, with the help of a table of random numbers. At Playa Cerritos a total of 592 were randomly selected during 20 mo and at Punta Perico a total of 596 specimens.

During sampling the water temperature was recorded, whether egg-capsules could be found and whether copulas were observed. Injecting a 10% neutral buffered formalin solution into the snails preserved the tissue of the animals. Subsequently about 24 h later in the laboratory the total SL of the animals was determined from the apex to the end of the siphon channel with the help of a digital caliper with a precision of 0.01 mm, the shell was broken and the gonads and the digestive gland were removed. Table 1 shows the number of gonads examined, the average total SL with standard deviation, and the size range. Sex determination was accomplished by visual inspection for a penis, and verified by histological examinations of the gonads.

#### Observations by Light Microscopy

The gonads were dehydrated in the alcohol series, cleared with butylated hydroxyanisole, and embedded in paraffin. The sections (7–9-µm thick) were prepared from the thickest part of the gonads, and subsequently, according to the methodology described by

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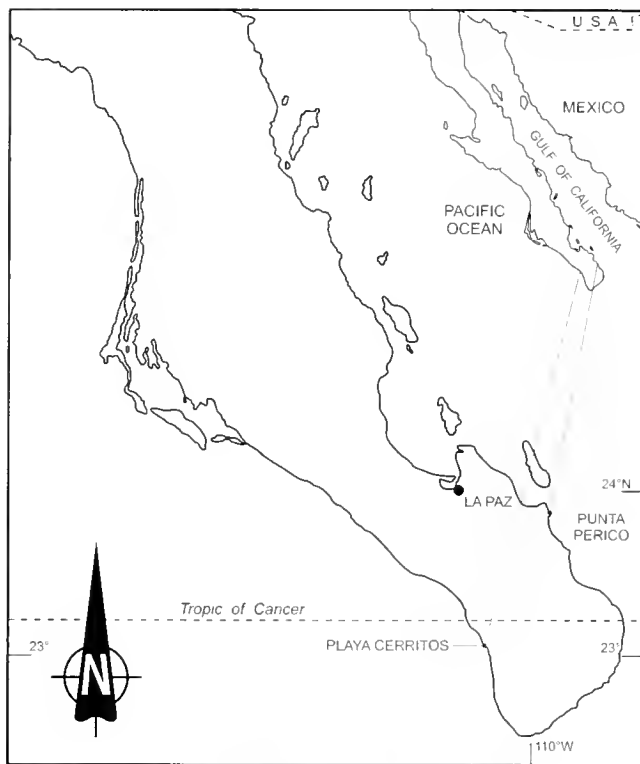


Figure 1. Map showing the study sites.

Luna (1968), stained with Harris hematoxylin and contrasted with eosin. This method was adopted after verifying in four specimens, that gonadal maturity was uniform in different parts of the gonad. Both sexes were confirmed by light microscopic examination of histological preparations of the gonads. Light micrographs were taken with a digital camera (Olympus Camedia C-5060) mounted on a light microscope (Olympus BX 41).

#### Reproductive Cycle With Gonad Developmental Phases

To determine the seasonality and duration of the reproductive cycle from the histological preparations the development of the gonads is classified arbitrarily: in females it is divided into four successive stages (developing, ripe, spawning, recovering), in males only into 3 (developing, spawning, and recovering) stages.

#### Developing Stage

In female gonads in the developing stage can be found oögonia and developing oocytes (Photo 1a). The size of the gonads is increasing through the accumulation of growing oocytes accumulating yolk. The oocytes are predominantly previtellogenic. In male gonads can be observed the spermatogenic follicles, spermatogonia, spermatocytes, and spermatozoa arranged in characteristic bands (Photo 2a).

#### Ripe Stage

In females, all follicles are filled with ripe vitellogenic oocytes of polygonal shape containing mature yolk granules (Photo 1b). No empty space was observed between the follicles. In males, the ripe stage cannot be distinguished from the developing stage, and is therefore omitted.

#### Spawning Stage

In females, because most oocytes in the follicles are discharged into the environment, the lumen of the follicles becomes essentially empty. Spawning ovaries are characterized by the presence of spaces between the free oocytes in the lumen (Photo 1c). In males, the lumen of the follicles is partially empty with spaces inside, and a decrease in the quantity of spermatozoa can be noticed. The spermatocytes remain at the follicular walls (Photo 2b). In this stage the spermatid ducts are filled with spermatozoa (Photo 2b and 2c).

#### Recovery Stage

In males and females, after spawning the gonads are collapsed, and each follicle is empty. In females few residual oocytes are present, being phagocytized by amoebocytes. The gonads are reduced to a thin layer of tissue adjoining the hepatopancreas (Photo 1d). No active oögenesis is evident. In males phagocytosis occurs of the residual spermatozoa by amoebocytes, and no active spermatogenesis are evident. However, not in all samples in the recovery stage are the gonads visible because they have been completely absorbed and therefore it is only possible to determine anatomically the sex of the animals by the presence or absence of a penis.

#### Mean Maturity Index

Grant and Tyler (1983) proposed using the term "maturity index" for examinations of gonad histological sections and restricting the use of "gonad index" to the quantitative analysis of relative gonad weight or size. We obtained the mean maturity index for each monthly sample, according to the method described by Seed (1976) and Kautsky (1982) for the bivalve *Mytilus edulis* (Linné 1758). The number of animals in each stages of maturity was multiplied by the numerical ranking of the stage (recovering = 1; developing = 2; ripe = 3; spawning = 4) and by dividing the sum of these products by the number of individuals in the sample. The index varies from one, if the female gonads of the entire population are recovering and 4 if the whole female population is spawning. In the male population, because the ripe stage is missing, the index will be 3 if all animals are in the spawning stage, and 1 if all animals are recovering.

#### Biological Minimum Size at Sexual Maturity

To determine the minimum size at sexual maturity we selected from all collected animals the 10 smallest females and males from each location and determined their stage of gonadal maturity.

#### Sex Ratio of the Histologically Examined Specimens

The total number of males and females collected at Punta Perico and Playa Cerritos was subjected to the  $\chi^2$  test to examine whether statistically significant differences exist between the numbers of the different sexes examined histologically.

## RESULTS

#### Copulation, Egg Capsule Deposition, and Water Temperature

During the collection of the snails we observed at Playa Cerritos and at Punta Perico year-round copulations, whereas egg capsules could be found on both sites only during February to May. During this period at Punta Perico water temperatures of



TABLE 1.

Punta Perico	Males				Females			
	Number	Avg. Total Length (mm)	Stand. Deviat. $\pm$ (mm)	Range (mm)	Number	Avg. Total Length (mm)	Stand. Deviat. $\pm$ (mm)	Range (mm)
June 2000	11	30.28	3.92	23.9–40.7	7	37.03	10.62	23–58.7
July	14	35.35	4.36	26.1–45.0	14	35.71	4.26	26.0–44.2
August	14	30.2	5.71	19.6–45.02	12	33.33	7.21	23.16–47.46
September	16	31.04	4.24	26.82–38.33	3	36.9	4.31	32.75–42.55
October	19	32.17	6.49	20.88–48.93	10	34.19	6.32	23.3–45.68
November	12	32.18	5.31	24.23–43.6	8	34.18	4.73	24.75–41.55
December	11	33.09	5.41	25.62–47.83	16	35.45	4.18	25.08–47.14
January 2001	19	30.84	4.48	17.63–41	9	31.25	4.4	19.2–40.03
February	19	31.73	4.56	25.11–41.01	11	38.11	2.86	21.95–44.59
March	20	30.35	5.75	22.67–43.68	10	33.57	4.36	21.99–42.37
April	18	29.17	5.56	20.82–47.42	12	33.62	7.96	21.17–50.33
May	20	31.59	5.87	22.8–45.82	9	34.87	6.58	23.36–49.22
June	20	27.51	7.37	18.76–44.03	15	36.22	8.91	18.44–50.38
July	15	27.42	3.7	21.84–34.63	10	33.48	5.29	16.37–45.11
August	11	29.64	6.05	22.48–45.21	15	30.19	3.27	24.51–37.17
September	15	29.58	4.95	19.95–39.02	9	32.88	4.77	25.9–40.53
October	12	30.12	5.57	20.83–40.36	19	32.29	5.18	22.45–46.31
November	18	29.36	5.27	17.49–39.46	13	27.75	4.64	19.09–36.88
December	19	30.01	4.9	20.49–40.05	9	33.92	4	23.13–40.37
January 2002	19	27.83	3.47	14.8–34.67	10	32.24	5.72	23.06–42.11
Total	322	30.38	5.43		221	33.64	6.00	

Playa Cerritos	Males				Females			
	Number	Avg. Total Length (mm)	Stand. Deviat. $\pm$ (mm)	Range (mm)	Number	Avg. Total Length (mm)	Stand. Deviat. $\pm$ (mm)	Range (mm)
June 2000	11	27.53	3.39	22.3–31.1	12	31.17	3.72	24.2–36.3
July	16	26.76	3.58	21.0–43.5	8	27.16	5.68	20.5–40.6
August	12	29.75	2.51	25.06–32.55	17	34.39	5.86	24.81–48.38
September	10	28.42	2.96	23.46–31.59	9	31.37	6.78	23.89–46.55
October	17	28.54	4.25	21.62–35.66	12	29.08	6.16	23.07–41.12
November	11	27.89	2.93	17.18–27.64	10	29.85	5.81	24.41–42.93
December	12	23.31	3.18	17.18–27.64	17	29.85	5.38	24.41–42.93
January 2001	15	25.28	2.8	19.01–29.05	15	29.71	5.83	21.97–44.96
February	11	26.17	4.82	19.01–29.05	7	29.12	6.2	21.1–41.77
March	16	24.86	4.36	18.31–34.73	14	27.51	6.69	18.84–41.71
April	14	28.05	3.91	21.25–35.38	15	28.43	4.68	23.08–42.47
May	12	29.25	4.33	23.53–40.81	16	29.36	2.69	23.97–33.78
June	19	26.48	3.81	20.74–32.76	11	29.64	4.58	20.28–36.45
July	17	23.4	3.07	18.56–29.4	12	26.54	3.57	21.7–34.17
August	14	25.58	4.7	18.34–36.44	17	27.32	5.53	20.65–38.31
September	14	21.5	3.28	15.87–35.82	13	25.14	5.39	13–33.42
October	19	23	3.8	14.86–28.4	12	25.83	4.33	16.71–31.52
November	15	24.52	2.33	19.84–28.57	14	28.81	7.14	15.94–41
December	14	24.63	2.69	18.98–29.95	17	28.28	5.71	15.88–38.61
January 2002	15	26.79	3.54	20.21–33.43	12	28.78	5.04	17.74–36.89
Total	284	25.93	4.11		260	28.92	5.62	

24 C to 26 C were measured. The water temperature in summer reached up to 32 C. At Playa Cerritos slightly lower water temperatures were measured: January up to May of 19 C to 20 C, and during summer up to 28 C (Fig. 2). No relation could be determined between the water temperature and the copulation and capsule deposition.

#### Color of the Gonads

Marked variations in the color of the gonads could be observed during different sampling dates. During September to October the

gonads were dark brown, and no sexual differences could be observed. In contrast from November until April the female gonads were yellow, whereas the male gonads were brown. The histological observation that the gonadal maturity is uniform in different parts of the gonad confirmed the uniform coloration of the formal-fixed gonads.

#### Sex Ratio

In this 20-mo study we examined histologically 544 gonads from snails collected at Playa Cerritos (260 females, 284 males)

## Females

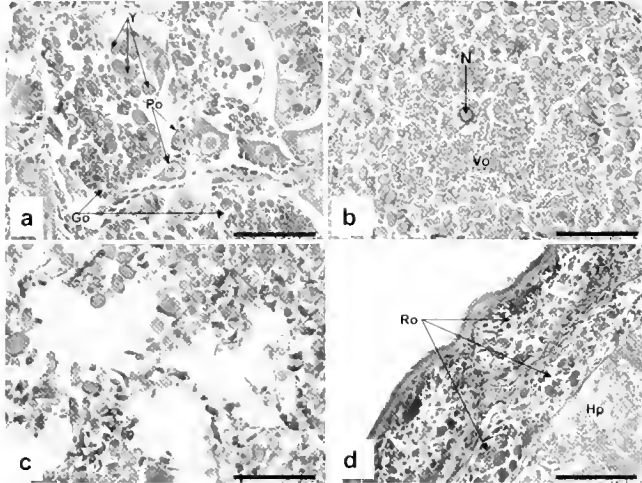


Photo 1. Light micrographs from histological sections of gonads from female *Plicopurpura pansa* snails. Scale bars = 100  $\mu$ m. (a) Developing stage: Yolk (Y), Previtellogenic oocytes (Po), Growing oocytes (Go). (b) Ripe stage: Vitellogenic oocytes ready to be released (Vo). (c) Spawning stage: Empty spaces and remains from broken can be observed. (d) Recovering stage: Residual oocytes (Ro), Hepatopancreas (Hp).

and at Punta Perico 543 specimens (221 females, 322 males). Applying the  $\chi^2$  test we examined the hypothesis that the sex ratio is 1:1. For the animals from Playa Cerritos the calculated  $P$  value was 0.3 showing that no statistically significant differences existed between the number of males and females. In contrast at Punta Perico were found statistically significant differences in the sex ratio ( $P < 0.001$ ). More males than females were found.

## Males

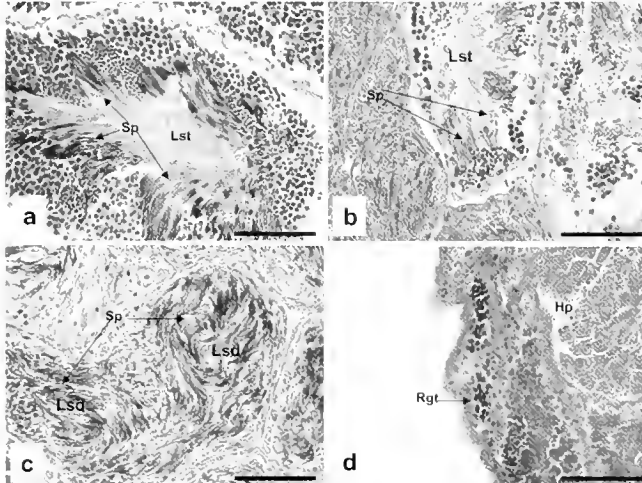


Photo 2. Light micrographs from histological sections of testis from male *Plicopurpura pansa* snails. Scale bars = 100  $\mu$ m. (a) Developing stage: Testis with different stages of spermatogenesis within the spermatid tubule. In the lumen of the spermatid tubule (Lst) can be observed spermatozoa (Sp) arranged in bands. (b) Spawning stage: In the lumen of the spermatid tubule (Lst) are few spermatozoa (Sp) visible. (c) Spawning stage: The lumen of the spermatid duct (Lsd) is filled with spermatozoa (Sp). (d) Recovering stage: Residual gonad tissue (Rgt), Hepatopancreas (Hp).

## Gonad Reproductive Cycle

## Punta Perico

In gonads from female snails collected at Punta Perico the developing stage was found nearly during the whole sampling period, with the exception of September to October 2000, and October 2001. In December 2001, 90% of the nine examined female gonads were in the developing stage. Female gonads were found in the ripe stage from June to August and November 2000, and from February to June 2001 and in January 2002, with the highest percentage in June 2000 (40% from 7 animals) and in February 2001 (36% from 11 animals).

Female gonads in the spawning stage were observed from June to August 2000, January to September 2001 and in January 2002. The highest frequency of spawning occurred in August 2000 and March 2001 (41% from 12, and 60% from 10 animals, respectively). Female gonads in the recovery stage were observed during nearly the whole sampling period, with the exception of June 2000 when no gonad was found in the recovery stage. In September to October 2000 and in October 2001, 100% of the 32 samples studied were in the recovery stage (Fig. 3a).

Male gonads from snails collected at Punta Perico were in the developing stage during the whole observation period, with two

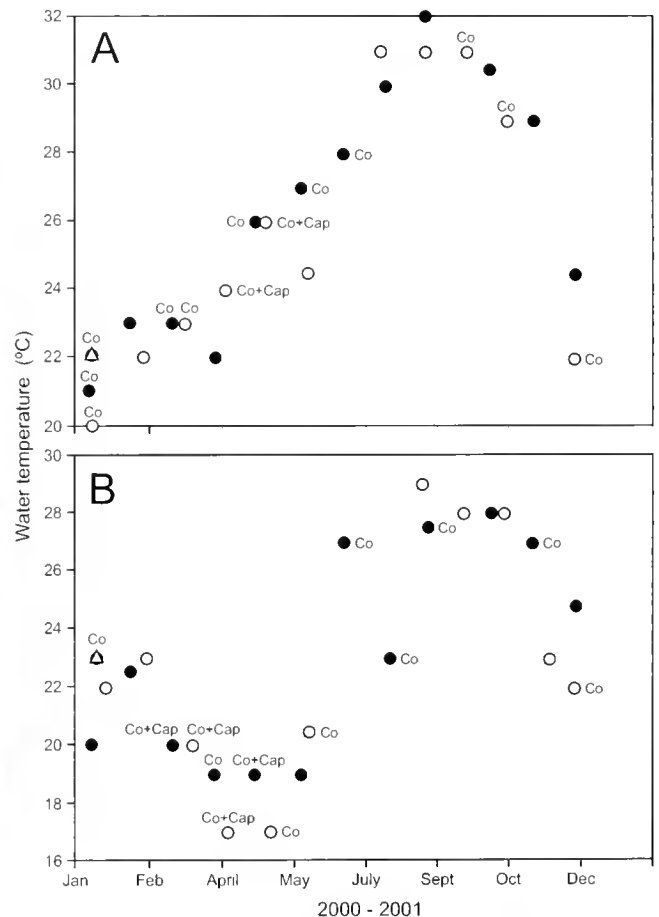


Figure 2. Copulation, egg capsule deposition of *Plicopurpura pansa*, and water temperatures at Punta Perico (A) and Playa Cerritos (B). Water temperature:  $\circ$ , 2000;  $\bullet$ , 2001;  $\triangle$ , 2002; Co = Copula; Cap = Capsules.

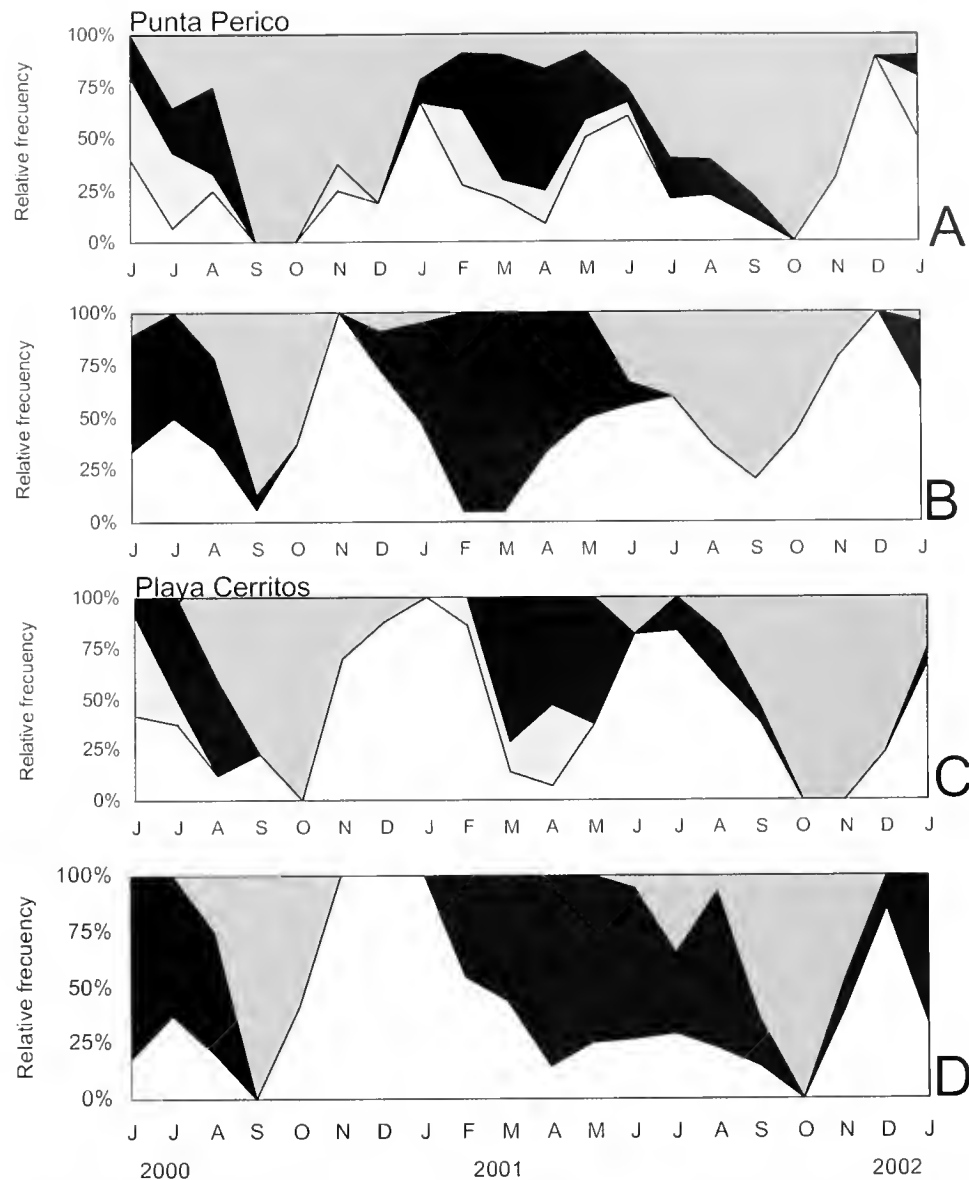


Figure 3. Male and female reproductive cycle from *Plicopurpura pansa* collected during the period from June 2000 to January 2002 at Punta Perico (A = females, B = males) and Playa Cerritos (C = females, D = males). □, Developing; ■, Spawning; ▤, Ripe; ▥, Recovering.

peaks of 100%: one in November 2000 (12 gonads studied) and another in December 2001 (19 gonads examined).

Individuals in the spawning stage were found during two clearly defined periods, one from June to September 2000, and another one from December 2000 until June 2001. Also in January 2002 the beginning of another spawning stage was seen. One spawning peak was found in February to March 2001 with 95% from a total of 39 male gonads studied. In September 2000, 87% of the 16 male gonads were examined in the recovery stage, and in September 2001 80% from a total of 15 gonads (Fig. 3b).

#### Playa Cerritos

Female gonads in the developing stage could be observed during the whole sampling period, with the exception of October 2000 and October to November 2001. Two peaks occurred, one in Janu-

ary and another in June to July 2001. In January 2001 all 15, and in June to July 82% from the 23 examined female gonads were in the developing stage.

The ripe stage of the female gonads showed two peaks of activity: one in June 2000 with 50% (12 gonads examined) and another in April 2001 with 40% from 12 examined gonads.

The spawning stage was observed between June up until August 2000 and March up until September 2001. In August 2000 from 17 examined gonads, 47% were in the spawning stage; in March 2001 from 14 examined gonads, 71% were in the spawning stage, and in August 2001 from 17 observed gonads, 24% were in the developing stage.

The recovery stage was observed during the periods: from August to December 2000 with a peak in October (100% from 12 examined gonads), and June 2001 to January 2002 with a peak in

October to November 2001 (100% from 26 examined gonads) (Fig. 3c).

The male gonads in the developing stage were observed for the whole sampling period, with the exception of September 2000 and during October 2001. Two peaks of activity were observed: in November, December 2000, and January from 38 examined male gonads were all 100%, and in December 2001 from 14 gonads 86%, in the developing stage.

Spawning took place from June to August 2000, with a peak in February 2001 (100% from 15 examined gonads), and from February to September 2001, with a peak in April 2001 (85% from 14 examined gonads).

The recovering stage was mainly observed from August to October 2000, with a peak in September 2000 when all (100%) of the 10 examined gonads was in the recovering stage. In 2001 the recovering stage was observed from July to November with peaks in September (64% from 14 examined gonads) and October (100% from 19 examined gonads; Fig. 3d).

#### Mean Maturity Index

Insight into the reproductive cycle of *P. pansa* was obtained from the combination of the monthly assessments of the gonad reproductive cycle and the monthly mean maturity index.

The mean maturity index for female gonads collected at Playa Cerritos had values above 2 during January to August, with peaks in February and March, demonstrating that the majority of the snail population was in the ripe and spawning stage. In September to October the majority of the gonads are found in the recovering

stage (Fig. 4b). The situation of the mean maturity index of the male gonads is very similar. From December to August the majority of the population is in the spawning stage, and from September to October the gonads are recovering.

The majority of the female gonads from snails collected at Punta Perico had during January to August a mean maturity index  $>2.0$ , demonstrating that they were, like at Playa Cerritos, in the ripe and spawning stage. An index value below 2.0, as observed from September to December, showed that they were in the recuperating stage (Fig. 4a). The same situation can be found in male gonads: the majority of the population was in the spawning stage during November to July, and only during August to October in the recovering stage. The data on the mean maturation index agreed well with the histological results described previously.

#### Size at Sexual Maturity

The 10 smallest female snails from the 260 females collected at Playa Cerritos had a total SL between 13.0–19.08 mm, and the size of the 10 smallest males from 284 animals SL ranged from 14.86–18.56 mm. In the females the gonad development stage was up to 18.77 mm SL, either recovering or developing. Spawning could be observed in females larger than 18.84 mm. In males, spawning could only be observed in animals larger than 18.00 mm. A similar situation could be observed from the snails collected at Punta Perico. The size of the 10 smallest females from 221 animals collected ranged from 16.37–21.00 mm and the size of the 10 smallest males from 322 animals ranged from 14.8–19.6 mm. Only one female of a size of 17.3 mm showed gonad development, larger animals were in the developing, ripe, spawning, or recovering stage. The gonads of the males were either in the developing (7) or in the recovering (2) stage. One male snail with a size of 18.94 mm had a gonad with signs of having spawned.

## DISCUSSION

#### Reproductive Cycle of *P. pansa*

The timing and duration of the reproductive cycle of *P. pansa* from gonad development through developing, ripe, spawning, and recovering is controlled by an interaction of environmental and endogenous factors. Activation of the gonads is probably controlled by endocrine hormonal factors being initiated in connection with lowered water temperature. With increasing air and water temperatures gonad development is starting and rapidly leading to maturation. Such effects were noticed by Chung et al. (2002) during studies of the reproductive cycle of the Korean muricid *Rapana venosa* (Valenciennes 1846). Kautsky (1982) made similar observations with the bivalve *Mytilus edulis*. Detailed information about the reproductive cycle of *P. pansa* is only sparingly available, mainly as thesis work, and it is difficult to compare one study with the other. Hernández-Cortés and Acevedo-García (1987) mention that copulation of *P. pansa* occurs at the Pacific coast of Oaxaca during the hot season from March until July, with the peak of occurrence in May, and from June to July the females are laying their egg capsules. Similar observations from Oaxaca are reported by Turok et al. (1988), who additionally mention that the peak of capsule deposition is in June, and that this continuous activity leads to a constant production of young animals during July, up until September.

In a histological study about the gonad development of *P. pansa* collected in Nayarit, Quiroz-Rocha (1992) reported

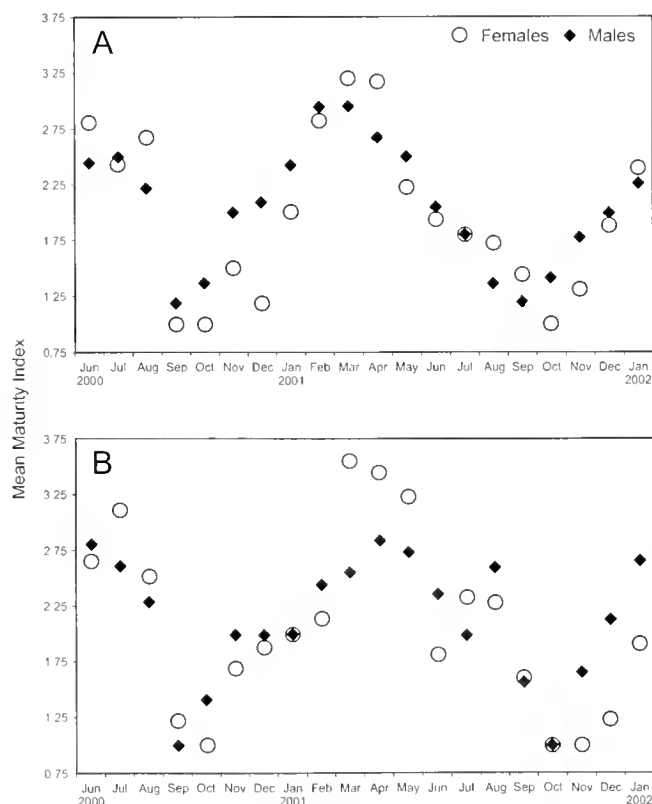


Figure 4. The mean maturity index for male and female gonads from *Plicopurpura pansa* collected at Punta Perico (A) and Playa Cerritos (B).

that during December to January the gonads are in the resting (immature) stage and that spermatozooids can be scarcely found in male gonads. During May to July the gonads are in the developing stage, and reaching the ripe stage during August to September. In a more detailed histological study about the gonad development cycle of *P. pansa* further north from Sinaloa, González-Flores (1997) reported that the recovering period occurs from September to October, but can be extended up until November to December. The developing stage can be observed in January to February but possibly can be extended during the whole year.

Copulation and the ripe stage of the gonads can be observed from March until July. Spawning starts in May and lasts until July. Acevedo-García et al. (1993) report, that females larger than 2 cm lay egg capsules during December up until September. In our study however, we could show that *P. pansa* can reproduce during the whole year with the highest frequency of spawning occurring in March and August. For adequate natural resources management of *P. pansa* in Baja California Sur, strict prohibitory measures should be enforced for exploiting animals during the developing, ripe, and spawning stages of the gonads, and only after special considerations should collections be permitted from September to October, when the animals are in the recovering stage.

#### *Influence of the Water Temperature on the Reproductive Cycle*

In most prosobranchs the reproductive cycle is in synchrony throughout a population, controlled by some kind of exogenous and/or endogenous controlling mechanisms (Webber 1977). Important exogenous factors for the gonad development of marine gastropods are considered to be temperature, nutrition, and photoperiod (Webber 1977, Martel et al. 1986). In temperate climates the seasonal changes of the temperature together with changes in the photoperiod are considered important factors controlling the gonad development, however in tropical zones where seasonal temperature fluctuations are less pronounced, temperature does not have such a marked impact. Many species have a seasonal spawning cycle spawn during springtime, and some even in winter (Webber 1977). In Argentina, in the southwestern Atlantic Ocean (Mar del Plata), *Zidonia dufresnei* (Donovan 1823) spawns during spring and summer (Giménez & Penchaszadeh 2002). In Coquimbo, North Chile, the subtidal gastropods *Priene scabrum* (King 1832) spawn in winter, and *Simun cymba* (Menke 1828) during almost the whole year (Romero et al. 2003). At the Pacific coast of Bahía Tortugas, Baja California Sur, Mexico, the wavy turban snail *Astrea undosa* (Wood 1828) spawns during the whole year, with spring and autumn as the major intensive spawning seasons (Belmar-Pérez et al. 1991). In Oaxaca Hernández-Cortés and Acevedo-García (1987), as well as González-Flores (1997) observed in Sinaloa the main spawning season of *P. pansa* is during the summer months of May to July. In Baja California Sur *P. pansa* has a spawning season from January to July until August, however, mainly in February to April during relative cold, and July to August during warm water and air temperatures. The gonad reproductive cycle of *P. pansa* seems therefore to be correlated with the temperature. The time of spawning of *P. pansa* might also be correlated with periods of decreasing differences between low and high tides. A decrease in the tidal range is observed in January to March and June to July up until September. During these periods the laid egg capsules are located either a short distance under the water surface or in water-splashed areas.

#### *Sex Ratio*

Differences in the sex ratio of a mature population of gonochoristic species can be used to check whether there is differential growth or mortality rate between the two sexes (Gimin & Lee 1997). In this study we examined the gonads from 284 males and 260 females collected at Playa Cerritos and no statistically significant difference was detected in the ratio between males and females. Also in Jalisco no difference in the male to female ratio could be found (León-Alvarez 1989). However, at Punta Perico a significant statistical difference in the male/female ratio (1:0.74) was observed. At this location we examined the gonads from 322 males and 221 females. The difference in the male/female ratio could be explained by the different conditions of the rocky shore at Playa Cerritos and Punta Perico. At Playa Cerritos, even at high tides, all the rocks are never completely submerged the water. In contrast, at Punta Perico, during high tides a great part of the shore is totally submerged and offering only limited hiding places to the snails, which need protection against displacement by the strong impact of the waves. To avoid the displacement by strong wave actions the snails prefer protective areas where they can hold fast onto the substrate (Hernández-Cortés & Acevedo-García 1987). Because females reach larger sizes, they are more endangered to be displaced by wave actions and currents than males (Denny et al. 1985). Although the reason is not mentioned, both Hernández-Cortés & Acevedo-García (1987) and Alvarez-Díaz (1989) reported that on the Pacific coast of Michoacan, males can be found in greater numbers than females (1: 0.83) and (1:0.75), respectively. Similar results were obtained by Turok et al. (1988) (1:0.78). Hernández-Cortés, and Acevedo-García (1987) in Oaxaca; and in Jalisco by Reyes-Aguilera (1993) (1:0.84), Fonseca-Madriz (1998) (1:0.72), and by Michel-Morfin et al. (2000) (1: 0.75). Another explanation for the occurrence of more anatomical males than females in a sample could be imposex, caused by the organic metal compound, tributyltin, which is used as a antifouling paint in shipyards, and which is leading to erroneous classification of males and females, resulting in a higher number of males (Gooding et al. 1999, Penchaszadeh et al. 2001). The comparison of our visual anatomical inspection of the animals with the histological examinations of the gonads show that the differences in the dominance of males at Punta Perico cannot be explained by imposex. The collection of the purple snail by fishermen as a bait and even as a specialty food could also be a reason for an unequal sex ratio. Fishermen prefer larger animals, and for this reason female snails are more sought after. In Punta Perico the population of *P. pansa* is not exploited by fishermen, and only environmental conditions could explain the unequal sex ratio observed there.

#### *Minimum Size at Sexual Maturity*

The determination of the minimum size at sexual maturity of any species of commercial interest is an important parameter for considerations of management and exploitation. Only fragmented results are available about the life history of *P. pansa*. After spawning, intracapsular development until hatching takes six to eight weeks at 22 °C (Naegel et al. 2003, Naegel 2004, Naegel & Gomez del Prado-Rosas 2004). The duration for settling and metamorphosis of the veliger larvae after spawning takes at least the same length of time (Naegel et al. 2003). The growth, environmental optimal range, and food requirements of the settled juvenile

niles smaller than 6 mm are as yet unknown. Certainly, for a snail to reach this size after spawning more than one year is needed. In this study we show that juveniles of *P. pansa* do not reach sexual maturity until they reached a size of more than 18 mm. It can be concluded that the purple snails begin to spawn when they reach a size of at least 18 mm. At this size they are at least two or three years old (Ramírez-Rodríguez & Naegel 2003). This extremely slow growth and long period before reaching sexual maturity could be caused by the harsh intertidal environmental conditions, such as the risk of desiccation and overheating through several hours of daily exposure to air, sun, and current reversal during the tidal cycle. Similar observations were made with the mangrove snail *Thais kioskiformis* (Duclos 1832), which showed a growth of only ~1 mm/year, and reaches the onset of sexual maturity at a SL of ~24 mm (Koch & Wolff 1996).

In view that *P. pansa* is in Mexico, because of over-exploitation in the past, today a species under special protection, and in view of the extremely slow growth rate, collecting snails <18 mm can potentially cause a drastic reduction in recruitment, and for this reason should not be permitted.

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## EFFECTS OF WATER TEMPERATURE ON THE LYSOSOMAL MEMBRANE STABILITY IN HEMOCYTES OF BLACKLIP ABALONE, *HALIOTIS RUBRA* (LEACH)

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**ABSTRACT** Neutral red retention (NRR) assay was used to evaluate the effect of changes in water temperature on lysosomal membrane integrity in the hemolymph of blacklip abalone, *Haliotis rubra*. Results from gradual temperature changes between 7 °C and 16 °C and between 16 °C and 25 °C showed that water temperatures within the range of 15 °C to 17 °C were optimal for maintaining lysosomal membrane integrity in this species. The rapid temperature changes between the ranges used in the gradual temperature change experiments indicated that when abalone were transferred directly between these temperatures their NRR time gradually increased or decreased to the level corresponding with the new temperature. However, when abalone were transferred directly between 7 °C and 25 °C or between 11.5 °C and 20.5 °C their NRR times initially decreased significantly, and then gradually increased to the levels corresponding with the new temperatures, indicating that different ranges of water temperature change can affect the lysosomal membrane integrity differently. The NRR times of blacklip abalone at 7 °C, 16 °C and 25 °C were  $40.0 \pm 2.89$  min,  $113.33 \pm 3.85$  min and  $35.0 \pm 2.89$  min, respectively.

**KEY WORDS:** neutral red retention assay, lysosomal membrane stability, temperature, blacklip abalone, *Haliotis rubra*

### INTRODUCTION

The blacklip abalone, *Haliotis rubra*, is a marine mollusc found in the southern waters of Australia (Shepherd 1975). This temperate species predominantly inhabits rocky inshore waters between 5 and 30 m in depth (Shepherd 1975), where the water temperatures range from 8 °C to 22 °C (Gilroy & Edwards 1998, Shepherd & Hearn 1983). This species is also economically important, worth millions of export dollars a year to Australia (Drew et al. 2001). During the last decade blacklip abalone aquaculture has developed substantially and has become one of the main abalone species farmed in Tasmania and Victoria. Most commercial operations in Australia use land-based raceway systems in the grow-out phase, where the water temperatures can drop to 7 °C in winter and increase to as high as 25 °C in summer (A. Butterworth pers. comm.).

The published data showed that some abalone species had little tendency to adapt to chronically altered thermal environments and little ability to withstand acute thermal shock (Gilchrist 1995). For example, Prince et al. (1987) found that the presence of warmer water in the north and northeast of Tasmania was the main reason for the stunted growth of the wild blacklip abalone in the region. Unusual high water temperatures in summer were suggested by Winstanley (1972) as one of the major factors causing high abalone mortality in some regions of Australia. Correlations between high seawater temperature and increased mortality were also established in the experiments with the Californian black abalone *H. cracherodii* (Steinbeck et al. 1992). Cheng et al. (2004) noted that when the Taiwanese abalone (*H. diversicolor supertexta*) was subjected to elevated water temperatures they were more susceptible to *Vibrio parahaemolyticus*.

Lysosomes are polymorphic, hydrolytic enzyme-containing organelles with many intracellular and extracellular roles (De Duve & Wattiaux 1966). In molluscs, one of the main functions of lysosomes is to digest macromolecules and break down damaged or old cell parts as well as destroy foreign invaders such as bacteria and viruses (Hauton et al. 2001). Neutral red retention (NRR)

assay measures the retention time of the neutral red dye in the lysosomes. In unstressed cells lysosomes will accumulate and retain the neutral red dye for an extended period of time. Once destabilized by a stress response the neutral red dye will leak into the cytosol of the cell through the damaged lysosomal membrane (Moore 1980, Pipe 1987, Lowe et al. 1995a, 1995b). As stressed cells lose their dye at a faster rate than nonstressed cells the rate of NRR in the lysosome can therefore be correlated to the overall stress of the animal (Harding et al. 2004a). Compared with other methods of measuring stress in molluscs NRR assay is convenient and inexpensive, thus this methodology is increasingly being applied as an index to evaluate environmental, physiological and mechanical stresses (Hauton et al. 1998, 2001). NRR assay has been used in assessing responses to various contaminants in mussels and freshwater snails (Lowe & Pipe 1994, Lowe et al. 1995a, b, Mamaca et al. 2005, Svendsen & Weeks 1994), to seasonal and environmental changes associated with the reproductive cycle, temperature, air exposure and food availability in mussels and oysters (Harding et al. 2004b, Zhang et al. 2006) and to mechanical disturbances related to grading, postharvest processing activities and storage conditions in mussels and oysters (Harding et al. 2004a, Zhang & Li 2006).

The main objective of this study is to investigate if NRR assay could be used to evaluate the effects of water temperature change on blacklip abalone *H. rubra* and their responding patterns in controlled experiments.

### MATERIALS AND METHODS

#### Experimental Animals

The abalone used in this study were obtained from a commercial farm located in Victoria and were approximately four years of age. Their height, length and width (mean  $\pm$  SE,  $n = 100$ ) were  $29.15 \pm 0.60$  mm,  $93.71 \pm 1.16$  mm and  $71.94 \pm 0.89$  mm, respectively. During the experimental period the abalone were held in 500 L tanks and fed on a standard artificial abalone diet provided by Adam & Amos Abalone Diets (Mt Barker, Adelaide, South Australia).

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### Neutral Red Retention Assay

The Neutral red retention (NRR) assay used in this study was modified from the methods used by Lowe et al. (1995a, 1995b), Hauton et al. (1998, 2001) and Zhang et al. (2006). The neutral red stock solution was made by dissolving 2.28 mg of neutral red powder in 1 mL of dimethyl sulphoxide (DMSO). The working solution ( $0.01 \text{ mg mL}^{-1}$ ) was prepared by diluting 17  $\mu\text{L}$  of the stock solution with 4 mL of artificial saline solution consisting of  $0.48 \text{ g L}^{-1} \text{ CaCl}_2$ ,  $1.45 \text{ g L}^{-1} \text{ MgSO}_4$ ,  $2.18 \text{ g L}^{-1} \text{ MgCl}_2 \times 6\text{H}_2\text{O}$ ,  $0.31 \text{ g L}^{-1} \text{ KCl}$ ,  $11.61 \text{ g L}^{-1} \text{ NaCl}$ , and  $0.35 \text{ g L}^{-1} \text{ NaHCO}_3$  (Buchanan et al. 2001).

Abalone hemolymph (0.2 mL) was drawn from the cephalic arterial sinus at the anterior end of the foot using a 29 G  $\times$  1-mL syringe. The hemolymph was then placed into a 2 mL siliconized Eppendorf tube containing 0.2 mL of artificial saline solution and gently mixed. Each abalone hemolymph sample was collected within 1 min to minimize the potential effects from handling. A 40- $\mu\text{L}$  mixture of the hemolymph sample and artificial saline solution was then placed onto a microscope slide treated with a poly-L-lysine solution (20  $\mu\text{L}$  poly-L-lysine in 100  $\mu\text{L}$  distilled water). The slide was then placed into a  $10^\circ\text{C}$  lightproof humidity chamber for 15 min to allow the cells to adhere to the slide. After 15 min the slide was removed from the chamber and the excess hemolymph was removed, 20  $\mu\text{L}$  of neutral red working solution ( $10^\circ\text{C}$ ) was then added to the cell layer and incubated in the chamber for another 15 min. A cover slide ( $22 \times 22 \text{ mm}$ ) was then placed onto the slide and the hemocytes were examined using a compound microscope ( $\times 400$  magnification). The slide was examined at 15 min intervals for the first 60 min and then every 20 min. A total of 50 granulocytes per individual were examined at each time interval. Once 50% of the hemocytes had started to lose dye from their lysosomes the assay was stopped and the time for the previous examination was recorded as the NRR time. The observer was not informed of the origin of the sample under examination to minimize the possibility of biased assessment.

### The Effect of Water Temperature Change on Lysosomal Membrane Stability

In this study each temperature treatment group was replicated three times by using three 500-L flow through tanks. Prior to each treatment 60 tagged abalone per replicate were acclimated at the required starting temperatures for seven days. At each sampling time hemolymph was taken from three randomly selected abalone in each replicate, with each of these abalone sampled once only.

#### Gradual Water Temperature Change

The gradual water temperature change experiment consisted of two treatments. In the first treatment, abalone acclimated at  $16^\circ\text{C}$  were split into three groups. The first two groups were respectively subjected to a gradual increase or decrease in water temperatures, whereas the third group was maintained at  $16^\circ\text{C}$  as a control. A temperature change rate of  $1^\circ\text{C}$  every two days was applied in the first two days and was changed to  $2^\circ\text{C}$  every two days thereafter until the final temperature of  $7^\circ\text{C}$  or  $25^\circ\text{C}$  was reached. In the second treatment abalone were also separated into three groups. In the first group abalone acclimated at  $7^\circ\text{C}$  were subjected to a gradual increase, whereas in the second group abalone acclimated at  $25^\circ\text{C}$  were subjected to a gradual decrease in water temperature until the final temperature of  $16^\circ\text{C}$  was reached in both groups. A temperature change rate of  $2^\circ\text{C}$  every two days was used until

$15^\circ\text{C}$  or  $17^\circ\text{C}$  was reached and then changed to  $1^\circ\text{C}$  every two days to the final temperature of  $16^\circ\text{C}$ . The third group was maintained at  $16^\circ\text{C}$  as a control. Hemolymph was sampled 48 h after the required temperature change. Once the final temperatures were reached the abalone were maintained for a further 6 days with hemolymph samples taken every 48 h during this period. The control samples were collected at each sampling time from the  $16^\circ\text{C}$  tanks.

#### Rapid Water Temperature Change

The rapid temperature change experiment consisted of four treatments. In the first treatment, abalone acclimated in  $16^\circ\text{C}$  water were split into two groups and transferred into  $7^\circ\text{C}$  and  $25^\circ\text{C}$  water respectively. In the second treatment two groups of abalone were acclimated at  $7^\circ\text{C}$  and  $25^\circ\text{C}$  respectively and then transferred directly into  $16^\circ\text{C}$  water. In the third treatment the first group was acclimated at  $7^\circ\text{C}$  and placed directly into  $25^\circ\text{C}$  water, whereas the second was acclimated at  $25^\circ\text{C}$  and placed into  $7^\circ\text{C}$  water. In the fourth treatment the abalone acclimated at  $11.5^\circ\text{C}$  were placed directly into  $20.5^\circ\text{C}$  water, whereas those acclimated at  $20.5^\circ\text{C}$  were placed directly into  $11.5^\circ\text{C}$  water. In each treatment the hemolymph was sampled at times 0, 0.5 h, 1.5 h, 3 h, 6 h, 12 h, 24 h, 48 h and 72 h and on days 5 and 7, respectively.

#### Statistical Analysis

All statistics were run with SPSS 13.0 software. The normality test was conducted first and showed normal distribution on all the data used in this study. ANOVA and Tukey *b* multiple comparisons were then applied. A probability level of  $P < 0.05$  was considered statistically significant.

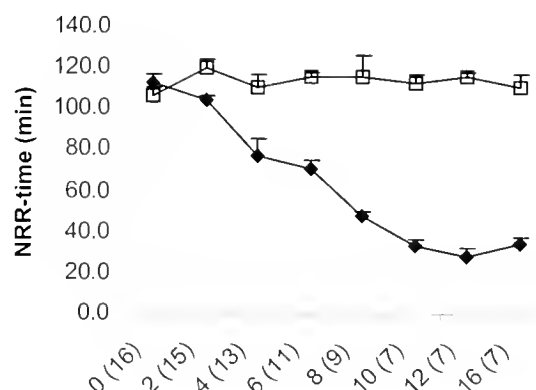
## RESULTS

#### Gradual Water Temperature Change

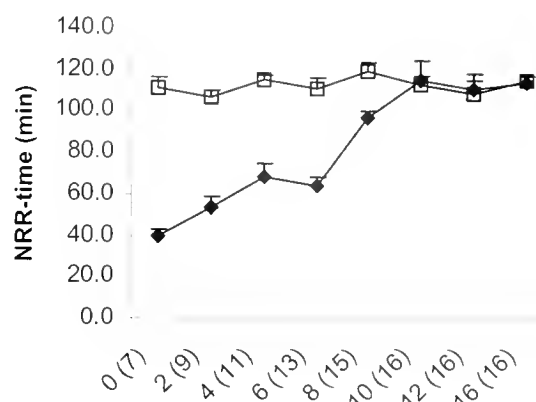
The results from the first treatment indicated that a gradual decrease or increase in water temperature from  $16^\circ\text{C}$  produced a declining trend in NRR time. When the water temperature was decreased from  $16^\circ\text{C}$  to  $7^\circ\text{C}$  (Fig. 1A) the NRR time did not change significantly ( $P > 0.05$ ) between  $16^\circ\text{C}$  and  $15^\circ\text{C}$ . The NRR time then dropped significantly ( $P < 0.05$ ) to  $77.78 \pm 8.01 \text{ min}$  at  $13^\circ\text{C}$ , and further decreased to  $33.33 \pm 3.33 \text{ min}$  on day 10 at  $7^\circ\text{C}$ . The NRR time then fluctuated at this level until the end of the experiment. When the water temperature was gradually raised from  $16^\circ\text{C}$  to  $25^\circ\text{C}$  (Fig. 1B) the NRR time did not change significantly ( $P > 0.05$ ) between  $16^\circ\text{C}$  and  $17^\circ\text{C}$ . Further water temperature increase from  $17^\circ\text{C}$  to  $19^\circ\text{C}$  produced a significant decrease in NRR time ( $P < 0.05$ ), dropping from  $108.89 \pm 5.88 \text{ min}$  to  $76.11 \pm 5.47 \text{ min}$ . The NRR time declined to  $31.67 \pm 1.67 \text{ min}$  on day 10 at  $25^\circ\text{C}$  and then remained at this level until the end of the experiment.

In the second treatment the gradual water temperature change from  $7^\circ\text{C}$  or  $25^\circ\text{C}$  to  $16^\circ\text{C}$  produced an increasing trend in NRR time. When water temperature was increased from  $7^\circ\text{C}$  to  $16^\circ\text{C}$  (Fig. 2A) the lowest NRR time of  $40.0 \pm 2.89 \text{ min}$  was observed on Day 0 at  $7^\circ\text{C}$ . The NRR time then increased gradually as the temperature was increased over time. At the temperature of  $15^\circ\text{C}$  on day 8 the NRR time reached a level that was not significantly different from the control ( $P = 0.352$ ). The NRR time increased slightly on day 10 once the temperature was changed to  $16^\circ\text{C}$  and remained at this level until the end of the experiment. When water

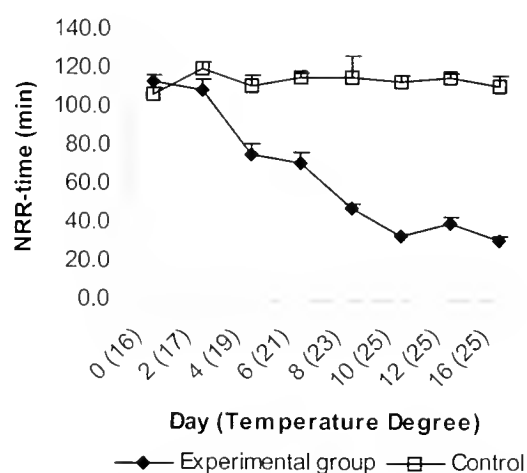
## A-Decreasing



## A-Increasing



## B-Increasing



## B-Decreasing

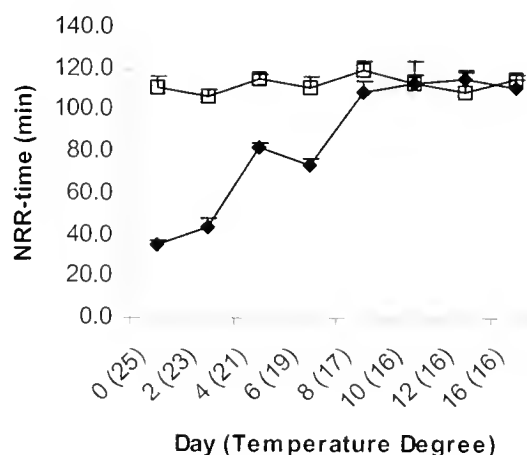


Figure 1. Comparison of NRR times in lysosomes of *H. rubra* with gradual change in temperature: (A) decreasing from 16°C to 7°C, (B) increasing from 16°C to 25°C. Bars express the mean + SE,  $n = 9$  abalone. Figures in brackets denote temperature (°C).

temperature was decreased from 25°C to 16°C (Fig. 2B) the lowest NRR time of  $35.0 \pm 2.89$  min was observed on day 0 at the temperature of 25°C. The NRR time then increased gradually as the temperature was decreased over time. On day 8 the NRR time was similar to the control ( $P = 0.848$ ) when 17°C was reached. The NRR time further increased slightly at 16°C and remained at this level until the end of the experiment.

During the experimental periods no significant difference in NRR times occurred in the controls in the two treatments ( $P > 0.05$ ).

#### Rapid Water Temperature Change

In the first treatment when blacklip abalone were transferred directly from 16°C to 7°C or 25°C water a rapid decline in NRR time was produced (Fig. 3). The NRR time dropped significantly ( $P < 0.05$ ) at each sampling time point within the first three to six hours from the initial level of  $113.33 \pm 3.85$  min at 0 h to  $50.0 \pm 2.89$  min at 3 h in the 16°C to 7°C group and to  $41.67 \pm 1.67$  min at 6 h in the 16°C to 25°C group. The NRR time then decreased

Figure 2. Comparison of NRR times in lysosomes of *H. rubra* with gradual change in temperature: (A) increasing from 7°C to 16°C, (B) decreasing from 25°C to 16°C. Bars express the mean + SE,  $n = 9$  abalone. Figures in brackets denote temperature (°C).

slightly to  $36.67 \pm 3.33$  min and  $31.67 \pm 1.67$  at 12 h in these two groups respectively. Statistical analysis showed that from 6 h to the end of the experiment the NRR times in each group did not differ significantly ( $P > 0.05$ ).

In the second treatment when abalone were transferred from 7°C or 25°C to 16°C the NRR times dropped from the initial levels of  $40.0 \pm 2.89$  min and  $35.0 \pm 2.89$  min at 0 h to  $30.0 \pm 2.89$  min and  $31.67 \pm 1.67$  min at 0.5 h, respectively (Fig. 4). From 0.5 h onwards the NRR time gradually increased over time. By 12 h the NRR times for the 7°C to 16°C and 25°C to 16°C groups were  $93.33 \pm 3.85$  min and  $93.33 \pm 3.85$  min, respectively, which was not significantly different ( $P > 0.05$ ) from the controls.

During the experimental periods no significant differences in NRR time occurred in the controls for the first two treatments respectively ( $P > 0.05$ ).

In the third treatment, when the abalone were transferred directly from 7°C to 25°C the NRR time dropped significantly ( $P = 0.014$ ) within the first 3 h from the initial level of  $40.0 \pm 2.89$  min

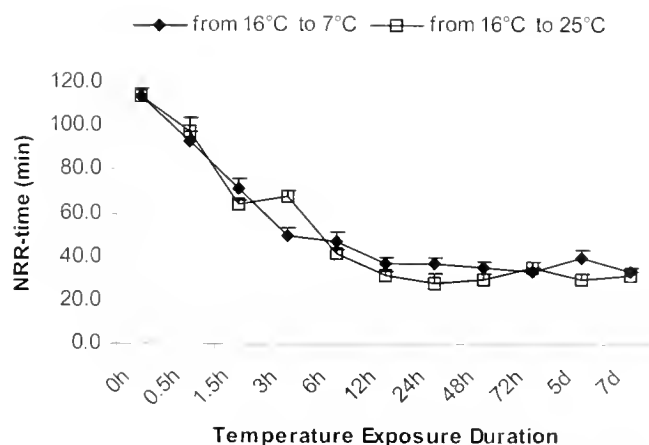


Figure 3. Comparison of NRR times in lysosomes of *H. rubra* with rapid change in temperature from 16°C to 7°C and 25°C, respectively. Bars express the mean + SE,  $n = 9$  abalone.

at 0 h to  $20.0 \pm 2.88$  min at 3 h and decreased further to  $16.67 \pm 1.67$  min at 12 h. From 12 h onwards the NRR time gradually increased to  $31.67 \pm 1.67$  min on day 7 (Fig. 5). When abalone were transferred directly from 25°C to 7°C the NRR time also dropped significantly ( $P = 0.022$ ) within the first 6 h from the initial level of  $35.0 \pm 2.88$  min at 0 h to the lowest level of  $18.33 \pm 1.67$  min at 6 h. From 6 h onwards the NRR time gradually increased to  $31.67 \pm 4.41$  min at 48 h and then fluctuated around this level until the end of the experiment. The mortality levels of the direct temperature changes from 7°C to 25°C and 25°C to 7°C were 24.6% and 17.5% respectively.

In the fourth treatment it was observed that the 11.5°C to 20.5°C temperature change group produced a significant drop ( $P < 0.05$ ) in NRR time from the initial level of  $77.78 \pm 5.88$  min at 0 h to  $38.33 \pm 4.41$  min at 1.5 h. The NRR time then increased gradually over the remaining sampling times, reaching  $73.33 \pm 3.85$  min on day 7 (Fig. 6). In the 20.5°C to 11.5°C temperature change group the NRR time dropped significantly ( $P < 0.05$ ) within the first 0.5 h from the initial level of  $73.33 \pm 3.85$  min at 0 h to  $38.33 \pm 1.67$  min at 0.5 h. From 1.5 h onwards the NRR time increased gradually and reached  $66.67 \pm 3.85$  min on day 7 (Fig. 6). No mortalities were recorded in this treatment.

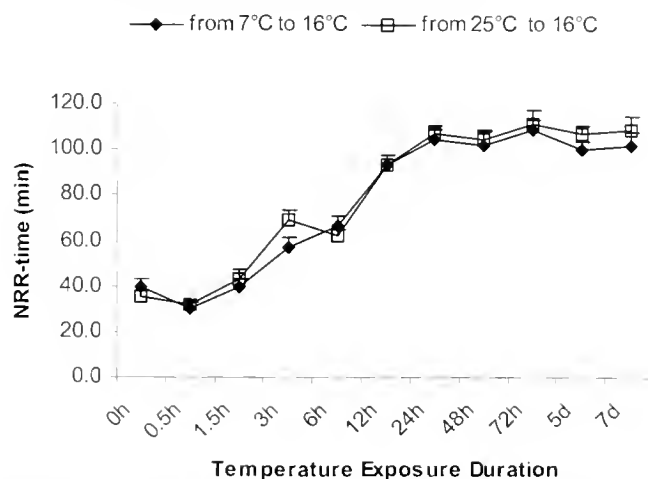


Figure 4. Comparison of NRR times in lysosomes of *H. rubra* with rapid change in temperature from 7°C and 25°C to 16°C, respectively. Bars express the mean + SE,  $n = 9$  abalone.

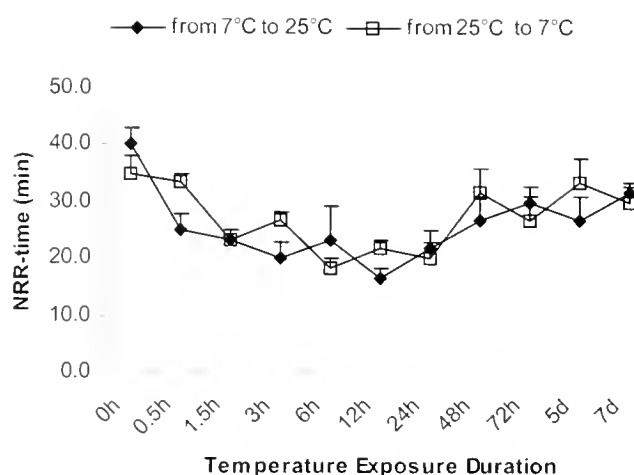


Figure 5. Comparison of NRR times in lysosomes of *H. rubra* with rapid change in temperature from 7°C to 25°C and 25°C to 7°C, respectively. Bars express the mean + SE,  $n = 9$  abalone.

#### The Effect of Rapid Versus Gradual Temperature Change on Lysosomal Stability at Final Temperature

Analysis of NRR times at 7°C, 16°C and 25°C indicated that the lysosomal membrane stabilities at these temperatures were not significantly affected by the speed (gradual or rapid) of change of water temperature ( $P > 0.05$ ).

## DISCUSSION

When water temperature was changed by 1°C from 16°C in the gradual temperature change experiments the NRR times changed slightly ( $P > 0.05$ ). As water temperature was further changed to lower than 15°C or higher than 17°C a significant drop in the NRR times was produced. These results indicate that temperatures between 15°C and 17°C are optimal for maintaining lysosomal membrane stability in blacklip abalone *H. rubra*. These results are also consistent with the findings in the published studies on this species by using performance and growth rate as indicators. Gilroy and Edwards (1998) found that the preferred temperature for *H. rubra* was 16.9°C and the optimum temperature for their growth was 17°C, which was supported by the finding of Harris et al., in

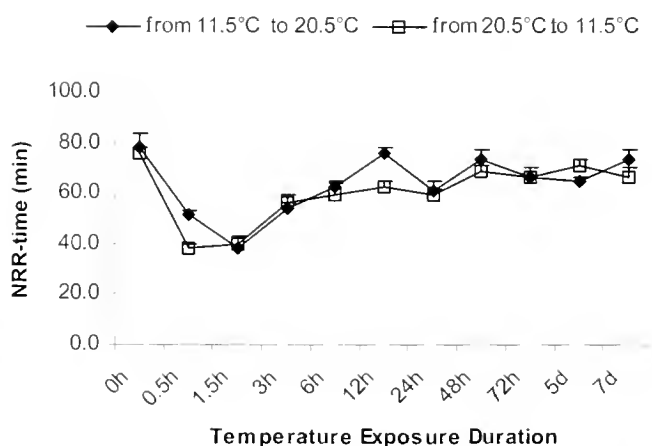


Figure 6. Comparison of NRR times in lysosomes of *H. rubra* with rapid change in temperature from 11.5°C to 20.5°C and 20.5°C to 11.5°C, respectively. Bars express the mean + SE,  $n = 9$  abalone.

2005 that the highest recorded growth for *H. rubra* was at the temperature of 17°C. Harris et al. (2005) also noted that at the temperature of 19°C the growth rate of *H. rubra* was reduced considerably.

The rapid temperature change experiment showed that when abalone were transferred directly from 16°C to 7°C or 25°C water the NRR times dropped to the levels that were not significantly different from those corresponding with 7°C or 25°C temperature within 3–6 h. When abalone were transferred from 7°C or 25°C to 16°C water their NRR times increased to the optimal level within 12 h. These results indicate that when *H. rubra* is subjected to rapid water temperature changes the lysosomal membrane destabilization is quicker than its stabilization. Similar results were also found by Zhang et al. (2006) with the Pacific oyster, *Crassostrea gigas*. In their experiments, when oysters were transferred from 15°C to 5°C or 25°C water, the NRR time decreased to the levels corresponding with 5°C or 25°C water within 3 h, which was substantially less than the five days required in the reverse experiment in which *C. gigas* was transferred from 5°C or 25°C to 15°C.

In the gradual and rapid temperature changes between 7°C and 16°C and between 16°C and 25°C the lowest NRR times were recorded at 7°C and 25°C, suggesting that these temperatures produced the greatest impact on lysosomal stability. However, no mortalities were recorded in these treatments, indicating that *H. rubra* could tolerate temperature changes in these ranges.

When blacklip abalone were subjected to the direct water temperature changes between 7°C and 25°C or 11.5°C and 20.5°C the NRR time decreased to the levels significantly lower than those previously recorded for these temperatures at the acclimation stages. It was also noted that after an initial decrease the NRR time increased to the levels corresponding with 7°C, 11.5°C, 20.5°C or 25°C water gradually, indicating that the lysosomal membrane had the ability to recover from this extra stress. In the between 7°C and 25°C treatments, a mortality level of more than 17.5% and a slower recovery in NRR times were recorded, suggesting that these temperature changes had produced the strongest physiological impact on the abalone in this study.

The differences between the start and the end temperatures were all 9°C in the rapid temperature changes between 7°C and 16°C, 16°C and 25°C and 11.5°C and 20.5°C. However, only the

between 11.5°C and 20.5°C treatments produced the extra stress response, suggesting that different ranges of water temperature change could affect the lysosomal membrane integrity differently.

The results from gradual and rapid water temperature changes between 7°C and 16°C and between 16°C and 25°C showed that in *H. rubra* the lysosomal membrane stability at the final temperatures of 7°C, 16°C, and 25°C were not significantly affected ( $P > 0.05$ ) by the rate (gradual or rapid) the water temperature was changed. A similar phenomenon was found by Zhang et al. (2006) in *C. gigas* at the final temperatures of 5°C, 15°C and 25°C.

In this study a logical correlation between water temperature changes and NRR levels has been established in blacklip abalone, *H. rubra*. In other publications high water temperature has been documented to affect the growth, health and survival in various abalone species (Prince et al. 1987, Winstanley 1972, Steinbeck et al. 1992, Cheng et al. 2004). It is, therefore, reasonable to expect that by using NRR assay as an indicator, the stress induced by temperature changes in some farming practices could be minimized.

Previous studies also showed that the performances such as growth rate of wild abalone were mainly influenced by temperature, food supply and channeling of energy into gonad production (Ino 1952, Sakai 1962, Poore 1972, Shepherd & Hearn 1983). However, these factors are often highly correlated and it is seldom possible to isolate causal effects between the seasonally changing components (Shepherd & Hearn 1983). It is anticipated that if the impacts of these factors could be quantified by a standard method such as the NRR assay used in this study their relative influences on the performances of wild abalone could then be evaluated. Comparison and prediction of abalone performances at different localities and/or seasons would then become possible. The response of NRR times to temperature changes in blacklip abalone has been characterized in this study. Further work is needed to determine the effects from other factors. Investigations with NRR assay on Pacific oysters have showed that starvation, gonad development and spawning could all individually significantly impair the lysosomal membrane stabilities in blood cells, thus imposing stresses on animals (Cho & Jeong 2005, Zhang & Li 2006, Song et al. submitted).

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## GROWTH AND DEVELOPMENT OF VEINED RAPA WHELK *RAPANA VENOSA* VELIGERS

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**ABSTRACT** Planktonic larvae of benthic fauna that can grow quickly in the plankton and reduce their larval period duration lessen their exposure to pelagic predators and reduce the potential for advection away from suitable habitats. Veined rapa whelks (*Rapana venosa*, Muricidae) lay egg masses that release planktonic veliger larvae from May through August in Chesapeake Bay, USA. Two groups of veliger larvae hatched from egg masses during June and August 2000 were cultured in the laboratory. Egg mass incubation time (time from deposition to hatch) ranged from 18–26 d at water temperatures between 22°C and 27°C. Four stages of rapa whelk veliger development along a time series from hatch to settlement were described using external morphological features. Rapa whelk veligers were measured at three-day intervals from hatching through the onset of spontaneous settlement. A four-parameter Gompertz growth model was used with length at age data from cultures of rapa whelk veligers to estimate maximum growth rates for June and August hatched groups. Larval rapa whelks settled at shell lengths of 1.18–1.24 mm after planktonic larval periods ranging from 24–42 d. Maximum larval growth rates observed in August ( $0.03 \text{ mm d}^{-1}$ ) are 50% lower than maximum larval growth rates observed for June hatched rapa whelk veligers ( $0.071 \text{ mm d}^{-1}$ ). Daily larval growth rates from hatch to first spontaneous settlement for rapa whelk veligers ranged from  $0.002$ – $0.099 \text{ mm d}^{-1}$  with maximum growth rates occurring between 12–18 d post hatch in June. Larval period duration, or the time from hatch to first spontaneous settlement, was 12 days shorter for June hatched larvae than for August hatched larvae. Water temperature was not significantly correlated with larval growth rates ( $\text{mm d}^{-1}$ ) in either June or August. Plasticity in larval period duration may enhance survival and dispersal opportunities for planktonic rapa whelk veligers.

**KEY WORDS:** veined rapa whelk, *Rapana venosa*, veliger, plankton, larval growth rate, propagule pressure, Muricid, gastropod

### INTRODUCTION

Many benthic invertebrates rely on planktotrophic development of larval forms as part of their life history strategies. Although planktonic larvae are vulnerable to pelagic predators and advection away from suitable habitats (Crisp 1974), planktonic development is a means to avoid benthic predators immediately after emerging from egg capsules and provides a mechanism for genetic exchange and dispersal (Scheltema 1971). Larvae with faster growth rates reduce their exposure to planktonic predators and dispersal vectors and potentially increase the number of larvae that survive to settlement. At the end of planktonic development, larvae settle to the benthos and spontaneously metamorphose losing specialized swimming structures in the process. The transition from pelagic to benthic lifestyle may be delayed in marine gastropods and the length of this delay may be related to the rate of larval development in the plankton (Pechenik 1980, Pechenik & Lima 1984), the presence of suitable settlement habitat (Crisp 1974, Pechenik 1980), and, in the case of larvae that use particular benthic prey, the presence of suitable prey items at settlement sites (Crisp 1974, Pawlik 1992, Davis & Stoner 1994).

Veined rapa whelks (*Rapana venosa*) are a recent addition to Chesapeake Bay's gastropod fauna. Native to Japanese and Korean waters, rapa whelk adults and egg masses were collected from Chesapeake Bay, USA in 1998 (Harding & Mann 1999). Native channeled whelks (*Busycotypus canaliculatus*), knobbed whelks (*Busycan carica*), Atlantic oyster drills (*Urosalpinx cinerea*) and Thick-lipped oyster drills (*Eupleura caudata*) all lay egg masses, which release fully developed juvenile snails that walk out of egg cases and begin benthic residence. In contrast, adult rapa whelks lay egg masses that release swimming veliger larvae, which develop in the plankton for 3–4 wk (Harding & Mann 2001) before eventually settling onto hard substrates, undergoing metamorphosis and taking up residence in the benthos. The period of plank-

tonic larval development may facilitate rapa whelk genetic exchange and dispersal into new habitats while reducing exposure to benthic predators prior to metamorphosis. The presence of a planktonic larval stage has enabled rapa whelk larvae to be moved across natural zoogeographic boundaries in ballast water of commercial ships and, within an estuary, may enhance dispersal and survival to recruitment with the end product being a net increase in propagule pressure, the rate at which breeding individuals are recruited to the population (Williamson 1996), and increased colonization success. The objectives of this study are to qualitatively describe rapa whelk veligers throughout their period of planktonic development and quantitatively describe growth rates of cultured rapa whelk larvae.

### MATERIALS AND METHODS

#### Egg Mass Sources and Culture

Adult rapa whelk broodstock were held in flow-through systems at ambient temperature and salinity conditions in the lower York River, Gloucester Point, Virginia with food (hard clams (*Mercenaria mercenaria*) and oysters (*Crassostrea virginica*), Harding & Mann 1999) available in excess.

During 2000, a subset of the egg masses produced by the broodstock populations in June and July were set aside for larval culture and description of larval development. Immediately after removal from the broodstock tanks, each egg mass destined for larval culture was placed in an individual container with 1 L of  $5\text{-}\mu\text{m}$  filtered seawater. The water in egg mass culture beakers was changed every other day. Egg mass incubation time (time from deposition to hatch or release of swimming veliger larvae) at ambient York River, Virginia conditions was recorded for each egg mass.

#### Veliger Culture

Egg masses that hatched on June 21 and 23 and August 1, 2000 provided rapa whelk veligers for descriptions of larval develop-

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ment and growth. After hatching or release of swimming veliger larvae from an egg mass, rapa whelk veligers were filtered from egg mass culture water using 80  $\mu\text{m}$  Nytex sieves and cultured in aerated filtered (5  $\mu\text{m}$ ) seawater (15–22 ppt) at temperatures of 25°C to 29°C and densities of <700 veligers  $\text{L}^{-1}$ . Veligers were fed a mixed algal diet (*Pseudoisochrysis* sp., *Chaetoceros* sp., *Tetraselmis* sp.) every other day and were maintained at 10 h dark/14 h light conditions.

#### Observations on Larval Development and Measurements of Larval Growth

A minimum of nine individual veliger larvae were removed from cultures and narcotized with a 7.5% magnesium chloride/seawater solution every three days from hatch (day 0) through the date of first natural spontaneous settlement of veligers within cultures. When veligers were sufficiently narcotized to stop swimming and rest on the bottom of the dish with vela extended and filtering, individual veligers were examined under a dissecting microscope to verify that they were alive and photographed with an image analysis system. After image capture, veligers were placed in 100% filtered seawater (equivalent to larval culture water), examined to verify that the effects of magnesium chloride had worn off, and returned to their original larval culture. Measurements of veliger shell length (SL, distance from the tip of the spire to the end of the siphonal canal to the nearest 0.001 mm) were made from the digital images using an image analysis system.

#### Data Analyses

Significance levels for all analyses were established *a priori* at  $P = 0.05$ . Assumptions of homogeneity of variance were tested with Bartlett tests and assumptions of normality were tested using the Ryan-Joiner test for normality.

Larval culture water temperatures from day of hatch to day of first spontaneous settlement for June and August hatched cultures were compared using a 1-way ANOVA with month of hatch as a factor. Temperature data satisfied assumptions of normality and homogeneity of variance without transformation.

Four parameter Gompertz models were used to describe rapa whelk larval growth in terms of shell length (mm) at age (d) data for presettlement and settlement stage rapa whelk veligers hatched in June and August using the equation:

$$L = L_0 + a e^{-e^{-\left(\frac{t-t_0}{b}\right)}}$$

where  $L_0$  is the veliger shell length (mm) at hatch or  $t = 0$ ,  $t$  is time posthatch or age in days,  $t_0$  is the time corresponding to the midpoint of the rise,  $a$  is the maximum attainable larval size, and  $b$  is a rate constant. Maximum growth rate during the larval period was calculated using the equation:

$$\text{Maximum growth rate (mm d}^{-1}\text{)} = 0.368 \left( \frac{a}{b} \right)$$

The Gompertz model has previously been used to describe molluscan growth on a length at age basis (Devillers et al. 1998, Chicharo & Chicharo 2000, Rodriguez et al. 2001). The four-parameter model was used because it incorporates larval size at hatching from the egg cases.

Larval growth rates at intervals during larval development were estimated for three-day intervals between measurements using the formula:

$$\text{growth rate (mm d}^{-1}\text{)} = \frac{\text{Average shell length (mm)}_{t+3d} - \text{Average shell length (mm)}_t}{3 d}$$

where  $t$  is the time post hatch or age in days. Average shell lengths were used because veliger growth rates were measured sequentially as groups randomly chosen from cultures instead of following individual veliger larvae for the duration of planktonic development. Linear regression analyses were used to evaluate the relationship between daily larval growth rate (mm) and water temperature (°C).

## RESULTS

In 2000, egg mass deposition in rapa whelk broodstock tanks began on May 15 and continued through August 15. Egg masses hatched June 21 through 23 were deposited between May 15 and June 6 with an average incubation time of  $26.3 \pm 2.3$  d (standard error) at average water temperatures of  $22.6^\circ\text{C} \pm 0.31^\circ\text{C}$ . Egg masses hatched August 1 were deposited on July 14 with an incubation time of 18 d at average water temperatures of  $27.2^\circ\text{C} \pm 0.18^\circ\text{C}$ . Chung et al. (1993) describe an incubation period of 17 d from deposition to hatch for rapa whelk egg cases from Korean waters in the laboratory at water temperatures of  $18.3^\circ\text{C}$  to  $20.4^\circ\text{C}$ .

For descriptive purposes, rapa whelk veligers along the continuum of development were assigned to one of four groups on the basis of visible morphological features. Davis et al. (1993) used a similar development scheme for three species of *Strombus* larvae. All references to age in days use the hatch date (0 d) as a starting point and are thus days-post hatch. Pictures of larval rapa whelk veligers are presented for all four developmental stages in Figure 1.

Stage I rapa whelk veligers (0–5 d) had a bilobed velum, a translucent foot and visible eye stalks (Fig. 1A). Their brown shells were smoothly rounded at the spire (Fig. 1A) with the development of the first whorl beginning 5–6 d post hatch. The beak and columella were visible at hatch and grew quickly, often presenting an almost translucent shell layer that gradually darkened with age (Fig. 1A, B). The beating heart was clearly visible through the shell at hatch. Two rows of cilia were visible along the outer edge of the velar lobes after 2 d (Fig. 1B). Veliger shell length at hatch estimates were between 0.406 (June) and 0.412 (August) mm (Table 1). Actual shell measurements were not made on veligers at hatch (0 d) because the rounded shell morphology made it impossible to position specimens (narcotized or not) on a single level plane under the microscope for image capture.

Stage II veligers (6–9 d) had an elongated beak, a four lobed velum with approximately proportionate lobes, and a relatively small opaque foot (Fig. 1C,D). By 6 d, one complete body whorl and shell shoulder were present (Fig. 1C) and the spiral line on the body whorl left by the presuture of the beak was obvious. Antennae were visible posterior to the eyestalks at day 9 (Fig. 1D).

Stage III veligers (10–23 d) continued to have an elongated beak with a suture in the middle and a proportionate four lobed velum but the foot was white and muscular with visible muscular contractions and greatly increased range of mobility (Fig. 1E,F). During Stage III, the foot began to have distinguishable anterior and posterior regions and became spade shaped. The operculum was clearly visible by 18 d. At 12–15 d, the larval shell was dark brown with two prominent shell shoulders, a well-developed col-



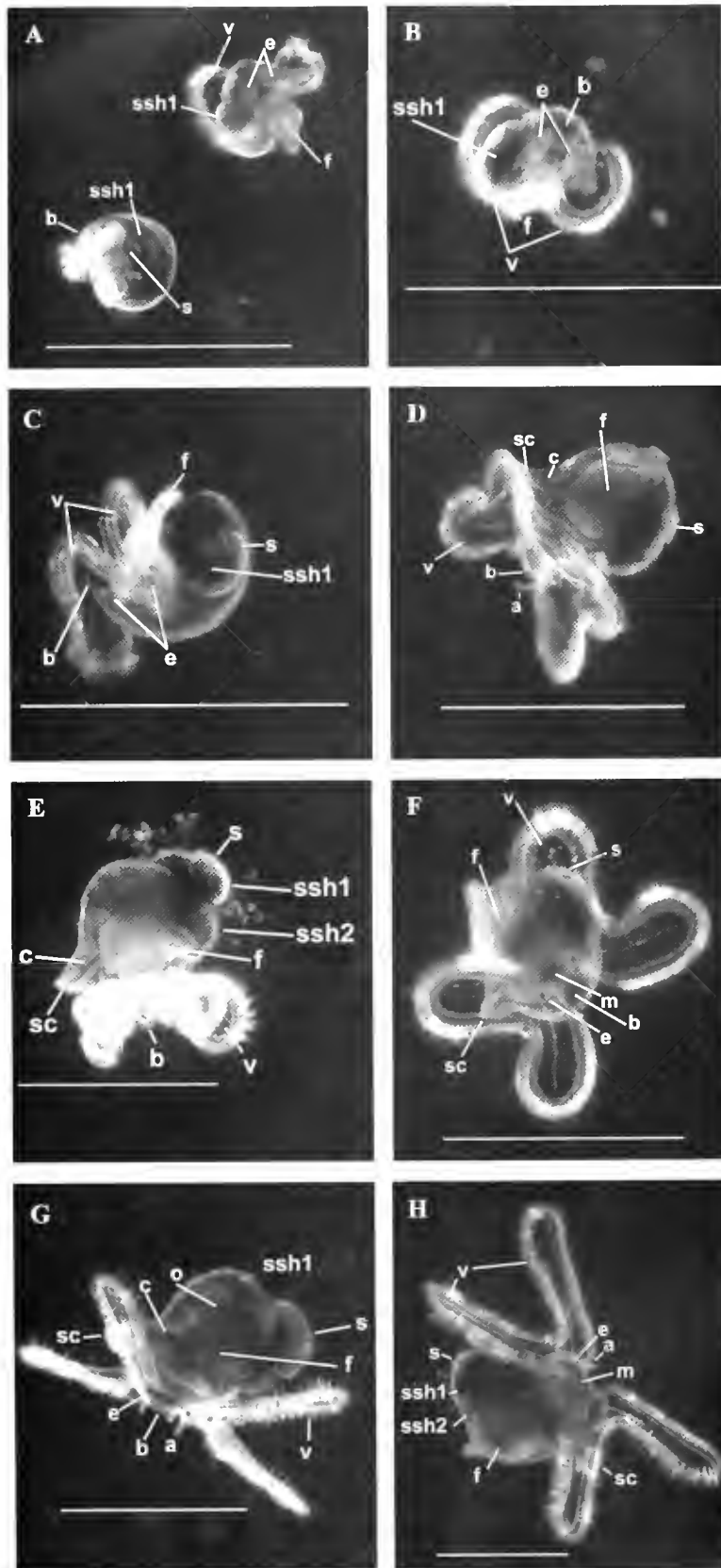


Figure 1. Pictures of rapa whelk (*Rapana venosa*) veligers in Stage I (A: 2-d-old veliger, B: 5-d-old veliger), Stage II (C: 6-d-old veliger, D: 9-d-old veliger), Stage III (E: 12-d-old veliger, F: 15-d-old veliger) and Stage IV (G: 38-d-old veliger, H: 23-d-old veliger). The scale bar in each picture represents 1 mm. Morphological features are noted, including the antenna (a), shell beak (b), columella (c), eye stalks (e), foot (f), mouth (m), operculum (o), siphonal canal (sc), first shell shoulder (ssh1), second shell shoulder (ssh2), spire (s) and velum (v).

TABLE 1.

Model coefficients for four parameter Gompertz regressions used to describe rapa whelk larval growth with standard error estimates for model coefficients in parentheses.

Hatch month	a	b	$t_0$	$L_0$	Model $R^2$
June	0.869 (0.044)	4.478 (0.538)	13.963 (0.376)	0.406 (0.023)	0.88
August	1.139 (0.153)	12.258 (1.967)	29.826 (1.718)	0.412 (0.014)	0.86

umella and siphonal canal, and visible rounded bumps/dimples on the exterior shell surface (Fig. 1E). By 18–21 d, a third shell shoulder was visible.

Stage IV veligers (24 d-settlement) had a prominent siphon that was typically extended from the siphonal canal and a four lobed velum where the anterior lobes were slightly larger than the posterior lobes (Fig. 1G,H). Veligers were competent to settle from 24 d forward, with size at settlement ranging from  $1.18 \pm \text{SE } 0.03$  mm (August) to  $1.24 \pm 0.03$  mm (June). By 27 d, three complete shell whorls were present with three distinct shell shoulders. All four lobes of the velum began elongating at 30 d becoming almost finger-like by 33 d. The foot continued to grow and became spade shaped (Fig. 1G,H). As the veligers aged, the beak gradually become less elongate and by 30 d was recognizable as only a simple point in swimming veligers. By 30 d, veligers spent at least half of the time with their velum retracted and the foot extended and probing available hard surfaces. At settlement, the shell was dark brown/black and the operculum had grown to the same size as the opercular opening and had dark brown/black edges with a light brown center.

Veligers hatched in June began settling at 24 d with all June cultures achieving settlement by 30 d. Veligers from all three August hatched cultures settled at 42 d. Larval culture water temperatures for June and August hatched animals were not significantly different (ANOVA,  $F = 0.65$ ,  $P = 0.42$ ).

Larval shell lengths increased rapidly between 9 and 21 d (Fig. 2A) for June hatched veligers. This time window corresponds developmentally and morphologically to Stage III when veligers had a 4-lobed velum, developing foot, and the shell attained its second shoulder or whorl (see earlier). Shell lengths for August hatched veligers increased more slowly throughout larval life (Fig. 3A) and August hatched veligers took 12 d longer to settle than June hatched veligers.

Maximum growth rates for June hatched veligers ( $0.071$  mm  $\text{d}^{-1}$ ) were twice as high as maximum growth rates observed for August hatched veligers ( $0.033$  mm  $\text{d}^{-1}$ ). Estimated daily larval growth rates from hatch to first spontaneous settlement for rapa whelk veligers ranged from  $0.002$ – $0.099$  mm  $\text{day}^{-1}$  and were generally higher during Stages I through III for June hatched veligers than for August hatched veligers (Fig. 2B and 3B). August hatched veligers were in Stage IV for longer than June hatched veligers and experienced higher relative growth rates than June hatched veligers at 24 and 27 d in Stage IV (Fig. 2B and 3B). It should be noted, however, that all June hatched veligers had settled by 30 d post hatch, whereas August hatched veligers did not settle until 42 d post hatch.

Larval culture water temperatures from June 21 and 23 (hatch dates) through July 15 (date of first settlement) ranged from  $25.1^\circ\text{C}$  to  $27.9^\circ\text{C}$  with a mean of  $26.6^\circ\text{C}$  (standard error  $\pm 0.17^\circ\text{C}$ ) with residual water temperatures typically within 1 degree of the mean (Fig. 2C). Larval culture water temperatures from August 1

(hatch date) through September 11 (42 d, date of settlement) ranged from  $25.3^\circ\text{C}$  to  $28.3^\circ\text{C}$  with a mean of  $26.6^\circ\text{C}$  (standard error  $\pm 0.18^\circ\text{C}$ ) with residual water temperatures up to  $1.5^\circ\text{C}$  of the mean (Fig. 3C). Water temperature was not significantly correlated with larval growth rates (mm  $\text{d}^{-1}$ ) in either June ( $df = 1$ ,  $F = 0.43$ ,  $P = 0.52$ ) or August ( $df = 1$ ,  $F = 0.10$ ,  $P = 0.75$ ).

## DISCUSSION

Larval rapa whelks settled at shell lengths of  $1.18$ – $1.24$  mm after planktonic larval periods ranging from 24–42 d. Maximum larval growth rates observed in August ( $0.03$  mm  $\text{d}^{-1}$ ) are 50% lower than maximum larval growth rates observed for June hatched rapa whelk veligers ( $0.071$  mm  $\text{d}^{-1}$ ). Dobberteen and Pechenik (1987) observed growth rates of  $0.027$  mm  $\text{d}^{-1}$  for 0–28 d old *Thais haemastoma canaliculata* maintained at  $24^\circ\text{C}$  in labo-

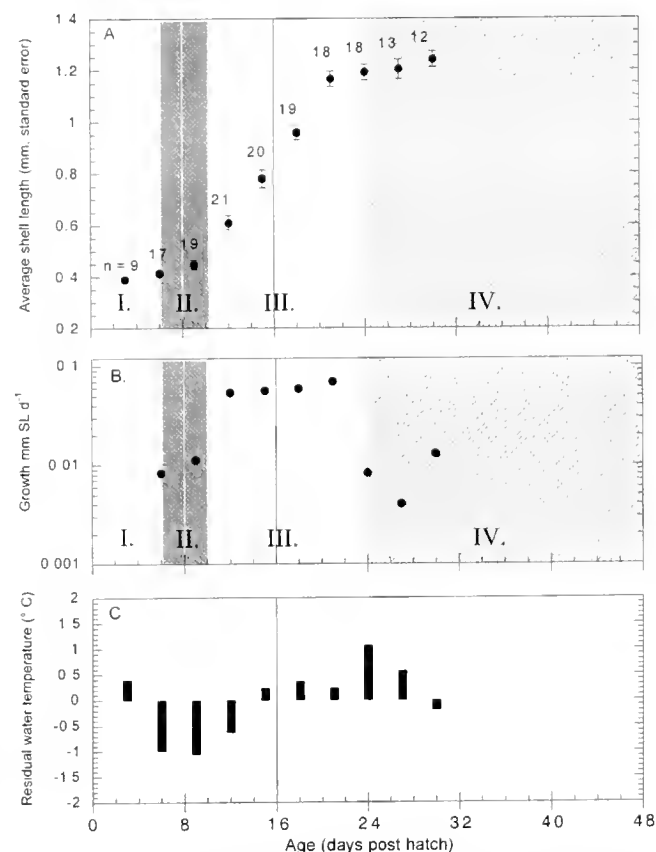
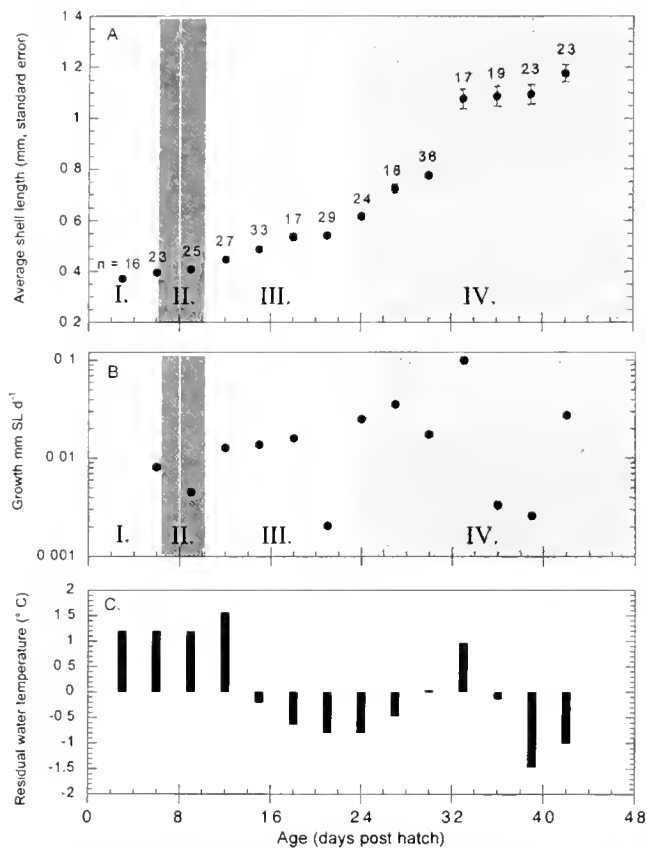


Figure 2. June hatched rapa whelk veliger (A) shell length (mm) at age (d) relationship, (B) growth rate (mm shell length  $\text{d}^{-1}$ ) through planktonic development and (C) larval culture water temperature residuals ( $^\circ\text{C}$ ) from the average larval culture water temperatures from hatch through 30 d.



**Figure 3.** August hatched rapa whelk veliger (A) shell length (mm) at age (d) relationship, (B) growth rate (mm shell length  $d^{-1}$ ) through planktonic development, and (C) larval culture water temperature residuals ( $^{\circ}C$ ) from the average larval culture water temperatures from hatch through 42 d.

ratory cultures. Daily larval growth rates from hatch to first spontaneous settlement for rapa whelk veligers ranged from 0.002–0.099  $mm\ d^{-1}$  with maximum growth rates occurring between 12–18 d post hatch in June. The differences in daily growth rates observed through development (this study) may explain the reductions in rapa whelk veliger survivorship and salinity tolerance observed by Mann and Harding (2003) at 12–18 d post hatch. The Stage III period in larval development is probably physiologically stressful because of the observed rapid shell growth. June hatched veligers approximately doubled their shell lengths during Stage III (12 d, 0.447 mm; 18 d, 0.958 mm). This trend was less noticeable for August hatched veligers whose overall growth rates were slower and settlement sizes were smaller.

Observed differences in growth rates and size at settlement between June and August hatched veligers may be related to culture conditions, parental physiological condition as expressed in the egg mass, or these differences may simply reflect a range of developmental plasticity within the species. Although average culture water temperatures ( $26.6^{\circ}C$ ) were the same for June and August larval periods, culture water temperatures were  $1\text{--}2^{\circ}C$  above average in early August for the first 6–9 d of larval development. Thermal tolerances of rapa whelk veligers are unknown, but slower growth rates early in larval life may have been related to thermal stress caused by warmer water temperatures. Rapa whelks lay multiple egg masses within a single breeding season (Chung et al. 1993, Harding & Mann 1999). In Chesapeake Bay, rapa whelks

begin laying eggs in May and egg laying continues through August (Mann & Harding 2003). The egg masses used herein were laid from mid-May to early June (June hatch) at the beginning of the adult egg mass laying period and mid-July (August hatch) towards the end of egg mass laying activity. Seasonal decreases in reproductive investment (as indicated by egg size or number per egg mass) have been documented for a marine polychaete (Qian & Chia 1992) and other marine molluscs (Ito 1997) with trends in decreasing number of eggs per clutch (egg mass) and egg size from the beginning to the end of the breeding season related to parental physiology and/or nutritional state. The egg masses used in this study were from multiple wild caught females. Information on adult condition before or at egg laying was not available. The position of each egg mass cultured for this study in the sequence of egg masses produced by each female in this breeding season is also unknown.

One of the most vulnerable portions of the rapa whelk life cycle is the period of planktonic larval development. Larvae that grow quickly in the plankton and reach competence quickly, reduce their exposure to planktonic sources of mortality. Relatively large size at competence increases postsettlement survivorship. Average rapa whelk shell length at settlement (1.18–1.24 mm) is similar to reported size at settlement for other gastropod larvae including *Thais kieneri* (1.04 mm, Middlefert 1996), *Strombina* (*Thais*) *haemastoma* (0.800 mm, Butler 1954), and *Strombus gigas* (1.170 mm, Davis et al. 1993). However, *R. venosa* size at settlement is approximately twice that of *Rapana rapiformis* (0.520–0.540 mm, Ramesh 1999) although both metamorphose and settle as early as 24 d post hatch (*R. rapiformis*, Ramesh 1999; *R. venosa*, this study). Moran (1999) predicts a positive relationship between growth, survivorship and size at settlement for intertidal gastropods. Large size at settlement brings with it increased resistance to starvation (Moran 1999) and reduced vulnerability to benthic predators (Spight 1976, Gossefin 1997).

The observed differences in larval growth rates and larval period duration may affect recruitment success of individual animals and invasion potential of the species as a whole. Animals that settle early in the year will be exposed to a longer growing season during their first year and, as a consequence, reach sexual maturity more quickly than animals that settle later in the year. From the standpoint of biological invasions, animals that grow rapidly to reproductive maturity increase the propagule pressure (Williamson 1996) within a habitat. The observed plasticity in rapa whelk growth rate and larval period duration resulting in successful settlement directly relates to the potential viability of larvae entrained and transported in ballast water as well as the survival of these larvae after introduction into new habitats. This plasticity may explain, at least to some degree, the continued success of rapa whelk invasions into new habitats. Recent invasions by rapa whelks include the Rio de la Plata, Uruguay and Argentina (Pastorino et al. 2000) and the Scheveningen, Netherlands (Vink et al. 2005). Historically, large long-lived gastropods like rapa whelks were not among the candidate fauna for anthropogenic transport to other habitats (Carlton 1999) and geographic range expansion by these benthic infauna relied on slow progression of individuals crawling into new areas or dispersal of planktonic larvae by water currents within the established range of ecological tolerances. Modern reliance on ballast water as part of commercial shipping offers a rapid vector for dispersal of the larval forms of these large predatory gastropods that supercedes natural zoogeographic boundaries and results in deposition of larval forms that have

evolved under one set of ecological and environmental constraints into new habitats lacking these traditional controls.

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## GASTROPOD ABUNDANCE AND BIOMASS RELATIONSHIPS WITH SALT MARSH VEGETATION WITHIN OCEAN-DOMINATED SOUTH CAROLINA, USA ESTUARIES

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**ABSTRACT** Molluscs, both gastropods and bivalves, appear to disproportionately influence the dynamics of salt marsh habitats. Bivalves typically are facilitators positively affecting the growth and survival of marshes, but gastropods may have unanticipated top-down effects through direct consumption of the predominant marsh vegetation, *Spartina alterniflora*. Natural, elevational differences in *S. alterniflora* density and morphology also may exert an influence on the spatial distributions of marsh snails. We examined the abundance of both plants and snails across an elevation gradient to determine if similarities or differences existed in the observed distribution patterns. Plant, mainly *S. alterniflora*, and snail, *Littoraria irrorata* (Say) and *Melampus bidentatus* Say, densities were measured along tidal creek to forest transects within the intertidal marshes of three marine-dominated South Carolina inlets. Significant differences in *S. alterniflora* density among marsh zones were paralleled by similar *L. irrorata* density differences. A consistent, unimodal pattern with peak *L. irrorata* density in the “short,” dense *S. alterniflora* mid marsh occurred within each inlet. In contrast, *M. bidentatus* was restricted almost entirely to the high marsh across all transects and inlets. Densities of *L. irrorata* and *S. alterniflora* were positively correlated in the low marsh consistent with the potential importance of culms as a food source and refuge from predators. *Littoraria irrorata* biomass was significantly different among zones in only one inlet and increased in a shoreward direction in two inlets. Although biomass patterns previously have not been reported for Southeastern US marshes, amounts were consistently greater than expected within the high-marsh zone. In SC, *L. irrorata* appears to be a conspicuous resident across the entire vegetated marsh and our results suggest previous studies focusing on the marsh near tidal creeks could underestimate appreciably the total population and overall significance of *L. irrorata* within coastal marshes.

**KEY WORDS:** *Littoraria irrorata* (Say), *Melampus bidentatus* Say, *Spartina alterniflora*, marsh zonation, snail biomass

### INTRODUCTION

Molluscs, both gastropods and bivalves, appear to influence disproportionately the dynamics of salt marsh habitats. Bivalves often are characterized as facilitators positively affecting the growth and survival of marshes either directly (Bertness 1984) or indirectly (Meyer et al. 1997, Dame et al. 2001). Early evidence suggested resident gastropods had limited to no direct effects on the salt marsh and primarily were involved in the recycling of detritus (Odum & Smalley 1959, Teal 1962, Stiven & Kuenzler 1979). However, recent studies indicate one of the common snail species, *Littoraria irrorata* (Say), has unanticipated top-down effects on the predominant marsh vegetation, *Spartina alterniflora* (Silliman & Zieman 2001, Silliman & Bertness 2002, Silliman et al. 2005).

The extent of potential gastropod effects on salt marsh environments ultimately will depend on the natural abundance and distribution of the various floral and faunal species. In Southeastern US marshes conspicuous plant zonation (see Chapman 1974) results from the interplay between physical tolerance to tidal inundation at lower elevations and interspecific competition at higher elevations (Pennings et al. 2005). Macrofaunal zonation patterns within marshes are less transparent (see however Kneib 1984, Netto & Lana 1997, Richardson et al. 1998, Angradi et al. 2001) with no accepted paradigm to explain observed patterns (Kneib 1984, Levin & Talley 2000). Ecological explanations for gastropod zonation in salt marshes could parallel paradigms established in the rocky intertidal (e.g., Connell 1961a, 1961b, Paine 1969). A result would be an overlap in the peak distribution of a possible keystone species, *L. irrorata*, and the maximum production of its primary food and habitat resource, *S. alterniflora*. To

establish if an overlap exists, we documented the zonation patterns of *S. alterniflora* and the two predominant gastropod species, *L. irrorata* and *Melampus bidentatus* Say, within three South Carolina inlets.

*Littoraria irrorata* and *M. bidentatus* are both common in western Atlantic salt marshes, but *L. irrorata* typically is reported from monospecific stands of *S. alterniflora* (e.g., Smalley 1959, Crist & Banta 1983). *M. bidentatus* is found in greater densities within the more speciose high marsh (Kerwin 1972, Fell et al. 1991). However, density, size and biomass have not been shown to vary in a consistent manner across the marsh for either species. Factors that may affect snail abundance along a tidal elevation gradient also have been implicated in affecting gastropod size distributions. Vermeij (1972) proposed intertidal gastropods would exhibit one of two possible patterns attributed to either predation or desiccation effects: Type 1 or a shoreward increase in size because larger snails are better able to resist desiccation and Type 2 or a decrease in size over the same gradient, because larger individuals would be less susceptible to aquatic predators. Littorinids in the rocky intertidal primarily exhibit a Type 2 pattern suggesting a response to predation pressure (Vermeij 1972). In saltmarshes, blue crab (*Callinectes sapidus*) predation on *L. irrorata* also decreases with increasing distance away from tidal creeks (Schindler et al. 1994, Lewis & Eby 2002). Although results from Schindler et al. (1994) and Lewis & Eby (2002) suggest *L. irrorata* should exhibit a Type 2 pattern, snail sizes have been shown to exhibit both Type 1 and 2 patterns (Hamilton 1978, Crist & Banta 1983). To indirectly examine the possible effects of desiccation and predation on salt marsh snails we measured all snail shell lengths to test whether saltmarsh snails conformed to either of Vermeij's (1972) hypothesized size distribution patterns.

Differences in snail abundance, size and biomass along a tidal elevation gradient in three ocean-dominated Southeastern marshes

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were examined to test whether physical or biological factors contributed to observed zonation patterns. Specifically, we hypothesized that *L. irrorata* density, biomass and mean length would vary in concert with vegetation zones because of a suspected dependence on *S. alterniflora* for food (Silliman & Bertness 2002, Silliman & Newell 2003) and increased aquatic predation in the low marsh (West & Williams 1986, Schindler et al. 1994, Lewis & Eby 2002). We also expected that *M. bidentatus*, given its small size, thin shell and pulmonate physiology would be more abundant in the mixed vegetation of the high marsh where desiccation and not predation would contribute most to the zonation pattern. Specifically, we set out to: (1) examine if *L. irrorata* and *M. bidentatus* exhibited distinct zonation patterns within southeastern US marshes; (2) evaluate existing size-frequency distributions to gain insights into possible mechanisms influencing salt marsh snail distributions and (3) examine if any relationship existed between the density and biomass of *L. irrorata* and the density of *S. alterniflora* stems.

## MATERIALS AND METHODS

### Study Site

Salt marshes in three ocean-dominated inlets along the northern South Carolina coast were selected for study: Hog, Murrells and North Inlets. Hog Inlet forms the southern boundary for Wailes Island (33°50'43"N, 78°35'12"W), a 0.5 × 4.0-km undeveloped barrier island. Study sites were in the extensive lagoonal marshes that exist along the backside of Wailes Island. Murrells and North Inlets have been studied extensively (e.g., Vernberg et al. 1992) but differ in residential development of the local watershed. Murrells Inlet, ca. 62 km south of Hog Inlet, has considerable shoreline and upland development although Huntington Beach State Park protects much of the southern portion of the Inlet. A study site within the mainland marshes just north of the park boundary near public oyster reefs was selected (33°31'17"N, 79°03'53"W). North Inlet is 31 km south of Murrells Inlet, and it is the site of the Baruch Marine Institute. The inlet and marshes are protected as part of the National Estuarine Research Reserve (NERR) system but a few residences and a golf course are located on the northern edge. Mainland study sites within North Inlet marshes were at Oyster Landing (33°31'04"N, 79°11'32"W) and Clambank (33°20'09"N, 79°11'36"W), two extensively studied sites (e.g., Morris & Haskin 1990).

### Sampling Design

To assess the spatial distribution of snails within the intertidal marshes without having to account for temporal differences, all samples were collected within a relatively short May to June period in 2003. The sampling period likely represents the time of greater snail density just after spring juvenile settlement and before increased summer mortality (KW, pers. obs.). Hog, North and Murrells Inlets were sampled three, two and one times respectively over the 2 mo. Snails were sampled along an approximate transect between tidal creek and forested upland uniquely established at each site on each date. A total of 5–20 samples from 0.25 m<sup>2</sup> quadrats placed along each transect within each of three vegetated zones was sampled. The low-marsh zone was identified by the presence of a monoculture of "tall" (>1 m) *S. alterniflora*. The short-form, <0.5-m tall, of *S. alterniflora* characterized the mid-

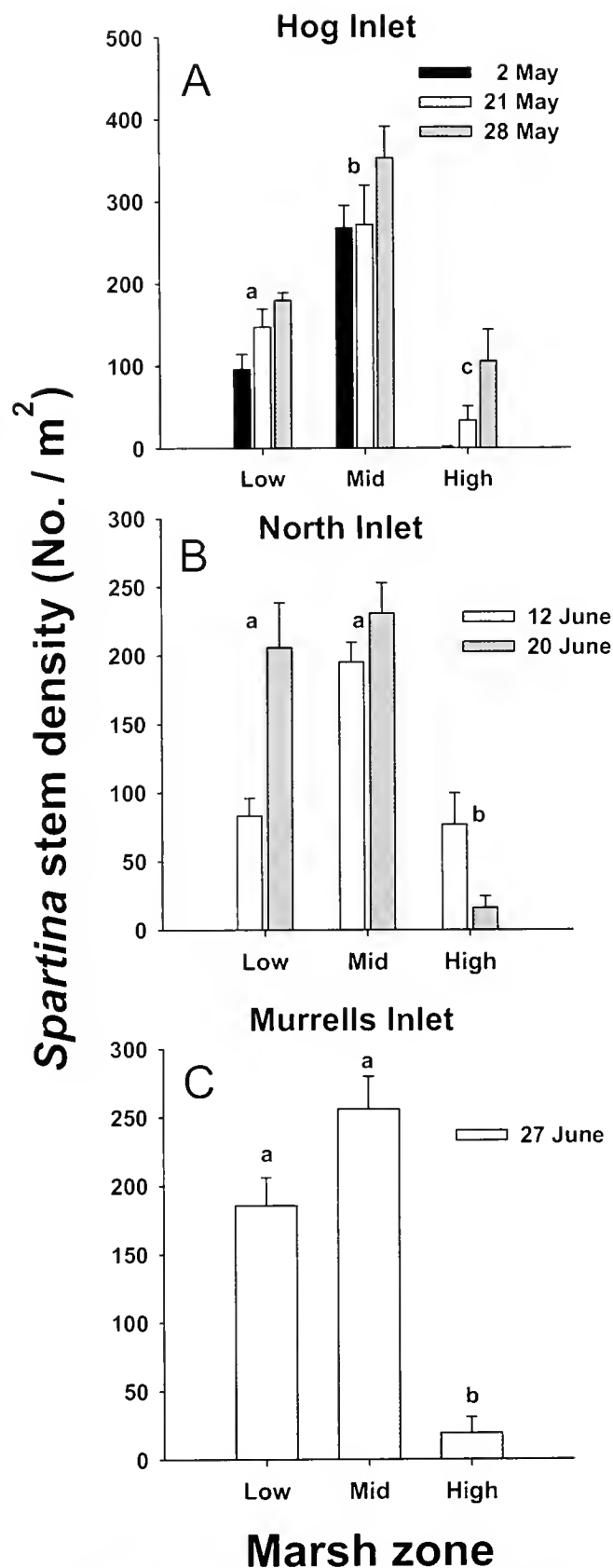


Figure 1. Mean ( $\pm 1$  SE) density of the total number of *Spartina alterniflora* stems in low-, mid and high-marsh zones of salt marshes in Hog Inlet (A), North Inlet (B) and Murrells Inlet (C). Different lower-case letters represent significant differences among zones.

marsh zone. Dense areas of *Spartina patens*, *Distichlis spicata* and *Juncus roemerianus* and the less common *Borrichia frutescens* and *S. alterniflora* identified the high-marsh zone at each site.

All snails within each quadrat were collected, returned to the laboratory and frozen at  $-20^{\circ}\text{C}$  for later counting, measurement and biomass estimation (see later). On the first sampling date, May 2, 2003, snails were counted and not collected; biomass was not estimated. The total number of *S. alterniflora* stems  $>10$  cm tall was also counted from a haphazardly chosen corner ( $0.25\text{ m} \times 0.25\text{ m}$ ) of each quadrat.

#### Laboratory Analyses

Snail biomass was estimated by measuring individual shell lengths and applying a length-mass regression for each species. Shell length was measured from the spire apex to aperture tip to the nearest 0.1 mm with digital calipers. To establish species' length-mass regressions shell length and tissue mass (without shell) were measured for a subset of snails collected from Hog Inlet sites ( $n = 184$  for *L. irrorata*,  $n = 56$  for *M. bidentatus*). Tissue was dissected from individual shells, dried to constant mass at  $60^{\circ}\text{C}$ , and ashed at  $500^{\circ}\text{C}$  for  $>4$  h in a muffle furnace to determine the ash-free dry mass (AFDM). A power curve was fit to the data for each species using nonlinear regressions in SigmaPlot (Vers. 8.0, SPSS, Inc.).

#### Statistical Analyses

Statistical differences ( $\alpha = 0.05$ ) among marsh zones and transects within inlets were assessed using analysis of variance (ANOVA) on  $\log_{10}(x + 1)$ -transformed values. Separate ANOVAs were conducted for each inlet using the General Linear Model procedure in SYSTAT (Vers. 10.0, SPSS, Inc.). Significant main effects were followed by Tukey HSD *post hoc* test to assess differences among levels (i.e., low-, mid and high-marsh zones). Any potential relationship between *L. irrorata* and *S. alterniflora* densities ( $\log_{10}(x + 1)$ -transformed) were examined with Pearson's Product-Moment correlation. The correlation between *L. irrorata* and *S. alterniflora* densities was calculated separately for each marsh zone using data from all inlets combined to assess overall relationships across inlets. *Melampus bidentatus* and *S. alterniflora* correlations were not considered because the distribution of *M. bidentatus* was restricted to the high marsh where little *S. alterniflora* was present.

The fit of *L. irrorata* to a Type 1 or Type 2 model (*sensu*

Vermeij 1972) within each inlet was tested by graphically examining the mean length of *L. irrorata* with respect to marsh zone. Juvenile snails  $<5$  mm were omitted from the analysis because individuals were typically found inside *S. alterniflora* leaf culms and likely were less susceptible to predation (Crist & Banta 1983).

## RESULTS

A significant power relationship between shell length and tissue AFDM was identified from the Hog Inlet subsample of both *L. irrorata* (tissue mass =  $0.0080 \times \text{length}^{3.1157}$ ,  $P < 0.0001$ ,  $R^2 = 0.928$ ) and *M. bidentatus* (tissue mass =  $0.0077 \times \text{length}^{2.4351}$ ,  $P < 0.0001$ ,  $R^2 = 0.930$ ). Snail biomass differences among zones and transects were estimated using these equations.

*Spartina alterniflora* stem densities in each inlet marsh exhibited a similar pattern: mid  $>$  low  $>$  high marsh (Fig. 1). Significant stem density differences existed among zones in each inlet, but they differed only among transects in Hog Inlet (Table 1). Stem density was always significantly lowest in the high marsh (Fig. 1).

*Littoraria irrorata* density was significantly different among marsh zones in each inlet (Table 1), and showed a similar overall pattern as *Spartina* density in each respective inlet (Fig. 1 and Fig. 2). *Littoraria* density was significantly less in the high marshes of North and Murrells Inlets, and significantly greater in the mid marsh of Hog Inlet (Fig. 2). In contrast, biomass of *Littoraria* was only significantly different among zones in Murrells Inlet (Table 1), where it was significantly less in the low marsh (Fig. 2). Furthermore, biomass tended to increase from low to high marsh in North and Murrells Inlets (Fig. 2). No significant differences in density or biomass of *L. irrorata* were found among transects in any inlet (Table 1).

In each inlet the distribution of *M. bidentatus* primarily was restricted to the high marsh (Fig. 3) and resulted in significantly greater density and biomass in the high marsh zone (Table 1, Fig. 3). Densities were also significantly different among transects at Hog Inlet (Table 1).

*Littoraria irrorata* density was positively correlated with *S. alterniflora* stem density for low- ( $P = 0.008$ ,  $R = 0.423$ ) but not mid or high-marsh zones ( $P > 0.05$ ).

The shell length of *L. irrorata* increased in a shoreward direction (Type 1 pattern) in both Murrells and North Inlets (Fig. 4). However, there was no obvious pattern in *L. irrorata* shell size at Hog Inlet (Fig. 4).

TABLE 1.

Results (*F*-values) from analysis of variance for comparisons among marsh zones and transects of total *Spartina alterniflora* density, and abundance and biomass of *Littoraria irrorata* and *Melampus bidentatus* in three South Carolina inlets. Asterisks represent level of significance: \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ . Data were  $\log_{10}(x + 1)$  transformed.

Parameter	df	<i>Spartina</i> Density	<i>Littoraria</i> Density	<i>Littoraria</i> Biomass	<i>Melampus</i> Density	<i>Melampus</i> Biomass
Hog Inlet						
Zone	2, 116 <sup>1</sup>	66.96***	27.79***	0.73	97.42***	320.93***
Transect	2, 116 <sup>1</sup>	17.89***	1.69	0.08	10.09***	0.28
North Inlet						
Zone	2, 32	17.43***	4.46*	1.78	121.74***	457.20***
Transect	1, 32	0.683	1.64	0.02	3.85	0.03
Murrells Inlet						
Zone	2, 12	15.65***	14.23***	24.42***	49.35***	101.68***

<sup>1</sup> Transect df = 1 and Error df = 63 for biomass of *Littoraria* and *Melampus* at Hog Inlet only.

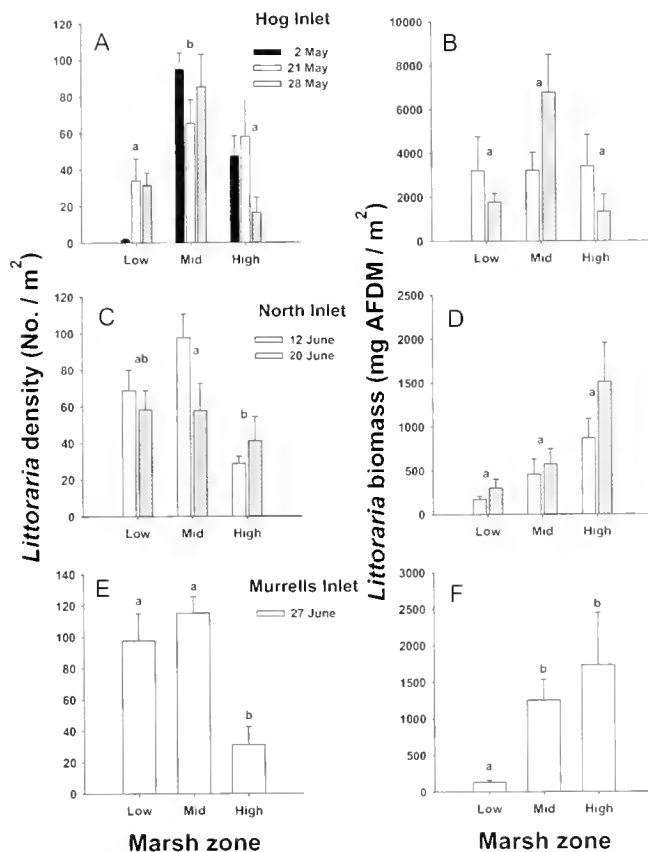


Figure 2. Mean ( $\pm 1$  SE) density of *Littoraria irrorata* in low-, mid and high-marsh zones in Hog Inlet (A), North Inlet (C) and Murrells Inlet (E). Mean ( $\pm 1$  SE) biomass of *Littoraria irrorata* in low-, mid and high-marsh zones of salt marshes in Hog Inlet (B), North Inlet (D) and Murrells Inlet (F). Different lower-case letters represent significant differences among zones.

## DISCUSSION

Significant differences in *S. alterniflora* density among marsh zones were paralleled by similar *L. irrorata* density differences. The same zonation pattern (mid > low > high marsh) in *S. alterniflora* stem densities occurred in each inlet and is common for western Atlantic salt marshes (e.g., Valiela et al. 1978, West & Williams 1986, Gallagher et al. 1988). Gradients in *S. alterniflora* densities often are attributed to nutrient limitation (Valiela et al. 1978) or soil salinity (Pennings et al. 2005, Silliman et al. 2005), but the actual mechanisms that produce the observed plant density patterns in Southeastern marshes are not understood completely. Variation in *S. alterniflora* stem density typically is counterbalanced by differences in stem height and diameter (low > mid > high) that result in greater cordgrass productivity within the low marsh (Gallagher et al. 1980, Wiegert & Freeman 1990).

The significant differences in *L. irrorata* density among marsh zones in each inlet coincided with the variation in macrophyte density. A significant shoreward increase in *L. irrorata* density from "tall" to "short" *S. alterniflora* zones occurred in this study at Hog Inlet and is reported in previous studies (Crist & Banta 1983, West & Williams 1986, Schindler et al. 1994, Lewis & Eby 2002). Previous *Littoraria* studies typically have not sampled high-marsh areas within close proximity to the terrestrial-marsh boundary. We found that *L. irrorata* density declined from the "short" *S. alterni-*

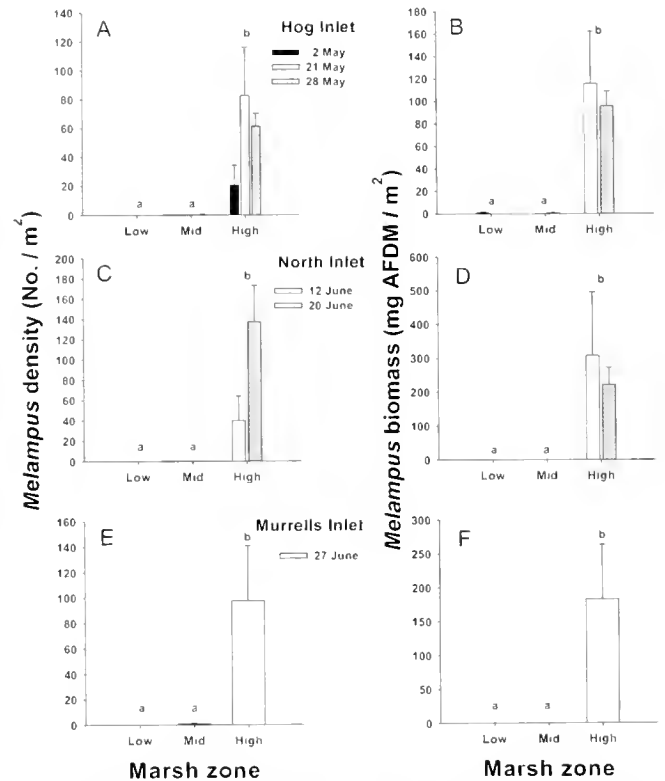


Figure 3. Mean ( $\pm 1$  SE) density of *Melampus bidentatus* in low-, mid and high-marsh zones of salt marshes in Hog Inlet (A), North Inlet (C) and Murrells Inlet (E). Mean ( $\pm 1$  SE) biomass of *Melampus bidentatus* in low-, mid and high-marsh zones of salt marshes in Hog Inlet (B), North Inlet (D), and Murrells Inlet (F). Different lower-case letters represent significant differences among zones.

*flora* mid marsh to the mixed-species vegetation of the high marsh. However, *L. irrorata* density was not significantly different between high- and low-marsh zones in either Hog or North Inlet. The greater than expected snail densities suggest that the high-marsh zone may be more productive than earlier studies suggest.

Unlike the patterns in snail density, significant variation in *L. irrorata* biomass across marsh zones only occurred in one inlet (Murrells). In two inlets (North and Murrells) a shoreward increase

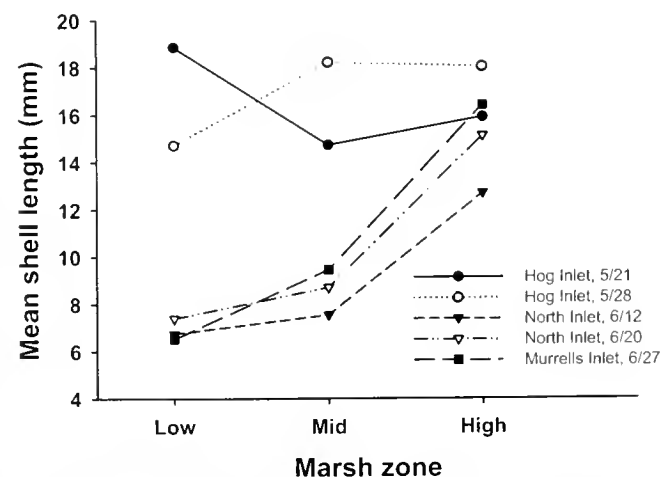


Figure 4. Mean individual length (mm) of *Littoraria irrorata*  $\geq 5$  mm in each zone and inlet.



TABLE 2.

Studies reporting mean densities (no./m<sup>2</sup>) of *Littoraria irrorata* and *Melampus bidentatus* within relatively unimpacted intertidal marsh habitats.

Study	Geographic Location	Marsh Zone	<i>L. irrorata</i> Density	<i>M. bidentatus</i> Density
This study	Hog Inlet, SC	Low	18	0
		Mid	85	<1
		High	41	51
This study	North Inlet, SC	Low	63	0
		Mid	74	0
		High	36	97
This study	Murrells Inlet, SC	Low	98	0
		Mid	115	1
		High	31	98
Cammen et al. (1980)	Walden Creek, NC	Entire	33	—
Crist & Banta (1983)	Wallops Island, VA	Entire	48	—
Newell & Barlocher (1993)	Sapelo Island, GA	Mid	460	—
Schindler et al. (1994)	Lighthouse marsh, GA	Low	22	—
		Mid/High	44	—
	Dean Creek, GA	Low	52	—
		Mid/High	66	—
	North End marsh, GA	Entire	52	—
Silliman & Bertness (2002)	Sapelo Island, GA	Low	5	—
		Mid	605	—
Silliman & Bortolus (2003)	Marshes from GA to VA	Low	0–5	—
		Mid	65–458	—
Silliman & Zieman (2001)	Hog Island, VA	Mid	48	—
Silliman et al. (2005)	Marshes from SC to LA	Healthy	1–558	—
		Die-off border <sup>2</sup>	15–2634	—
Stiven & Hunter (1976)	Russell's Creek, VA	Entire	108–135	—
	Ft. Macon, VA	Entire	70–92	—
Stiven & Kuenzler (1979)	Tar Landing marsh, NC	Entire	13	—
	Causeway marsh, NC	Entire	1	—
Warren (1985)	Wakulla Beach, FL	Entire	300	—
West & Williams (1986)	Dauphin Island, AL	Low	65	—
		Mid	145	—
Bishop & Hackney (1987)	St. Louis Bay, MS	Entire	—	6
Fell et al. (1982)	Branford Marsh, CT	Low	—	1–381
		Mid	—	556
		High	—	605–1222
Fell et al. (1991)	Barn Island, CT	Entire	—	392
Joyce & Weisberg (1986)	Canary Creek, DE	Entire	—	449–1234
Kerwin (1972)	Poropotank River, VA <sup>1</sup>	Entire	—	7

<sup>1</sup> Salt marsh sites only.<sup>2</sup> Border between healthy marsh and *S. alterniflora* die-off zones.

in biomass contrasted with the midmarsh peak in density (Fig. 2). Zonation patterns for *L. irrorata* biomass previously have not been reported, but patterns in mean shell length have been examined (Hamilton 1978, Crist & Banta 1983, Lewis & Eby 2002). Shell length increased in a shoreward direction in one study (Hamilton 1978) and decreased in another (Crist & Banta 1983). Patterns in shell length also can vary within a study, increasing shoreward in a marsh grazed by horses but decreasing in an ungrazed marsh (Lewis & Eby 2002). In both Murrells and North Inlets shell length increased shoreward corresponding to the patterns in snail biomass, but Hog Inlet snail lengths exhibited no clear trend (Fig. 4). Existing data on patterns in *L. irrorata* shell lengths are inconsistent with Vermeij's (1972) gastropod size-gradient framework: snails fit Type I and II patterns depending on the study and site. The extensive geographical range, from Virginia's Eastern Shore (Crist & Banta 1983) to Florida's Gulf Coast (Hamilton 1978), and resultant biotic (e.g., predation pressure) and abiotic differences

(e.g., tidal range) likely complicate interpretation of any shell length patterns.

Recent focus on the importance of top-down regulation of *L. irrorata* densities and ultimately salt marsh dynamics in the Southeastern US (Silliman & Zieman 2001, Silliman & Bertness 2002, Silliman et al. 2005) suggest possible mechanisms underlying any across marsh difference in snail density and size. Several studies document the effects of blue crab (*Callinectes sapidus*) predation on *L. irrorata* and the roles of tidal inundation and *S. alterniflora* density on snail predation (Hamilton 1976, Warren 1985, West & Williams 1986, Vaughn & Fisher 1988, Schindler et al. 1994, Hovel et al. 2001, Lewis & Eby 2002). Blue crab predation on snails is more pronounced closer to tidal creeks because of the increased inundation frequency and greater coverage times permitting typically subtidal predators to spend more time foraging and feeding (West & Williams 1986, Schindler et al. 1994, Lewis & Eby 2002). Along with fewer and shorter inundations in the mid

marsh, greater *S. alterniflora* stem densities hinder blue crab foraging (West & Williams 1986, Schindler et al. 1994, Lewis & Eby 2002). *Spartina alterniflora* can also provide an aerial refuge from predators once snails climb the stems (Hamilton 1976, Warren 1985, Vaughn & Fisher 1988, Hovel et al. 2001). The positive correlation between *L. irrorata* and *S. alterniflora* densities in the low-marsh zone of all inlets supports the use of stems as an important vertical refuge from predators. Similarly, West & Williams (1986) document a strong positive relationship between snails and stem density.

In contrast to *L. irrorata*, the distribution of *M. bidentatus* was restricted to the high marsh across all transects and inlets. In more northern marshes *M. bidentatus* also occurs primarily in the high-marsh, but they can also extend into the midmarsh or short-*Spartina* zone (Kerwin 1972, Price 1980, Fell et al. 1982). Our density estimates for *M. bidentatus* were often lower than those in northern Western Atlantic marshes (Table 2). The lack of any appreciable *M. bidentatus* outside of the high marsh in our study may be attributed to differences in soil salinity that result from latitudinal variation in evapotranspiration (Pennings & Bertness 1999). Similar to *L. irrorata*, *M. bidentatus* is also vulnerable to marine predators (e.g., especially *Fundulus heteroclitus*) moving onto the marsh during high tides and having a greater effect in the more frequently inundated low- and mid-marsh zones (Vince et al. 1976, Joyce & Weisberg 1986). Whether physically limited or biologically regulated to living a mostly terrestrial existence in the upper intertidal region, pulmonate *M. bidentatus* must remain in close proximity to areas of the marsh inundated by spring tides to enable development of its planktonic larvae (Russell-Hunter et al. 1972).

Snail densities for *L. irrorata* within Hog, Murrells and North Inlets were very similar to means reported in most other studies, but they were much lower than in some marshes further south, especially at Sapelo Island, GA (Table 2). A preference by *L. irrorata* for regions of higher salinity (as a result of increased

evapotranspiration) is a possible explanation, but our study inlets are ocean-dominated and some density estimates from marshes further south are similar to our own. Cross-site comparisons of population-limiting factors (e.g., predator populations, food resources, estuarine conditions experienced by veliger larvae) would be an essential step toward understanding these large differences in density among marshes.

Although variation in *L. irrorata* density has been the focus of several studies (Table 2), biomass of *L. irrorata* has seldom been estimated. In fact, none of the studies listed in Table 2, other than our own, examine variation in biomass within and among marshes. Given that most studies have found *L. irrorata* density peaking in the mid marsh, we expected biomass to show a similar pattern. Instead, the biomass of *L. irrorata* in the high marsh was unexpectedly high compared with the other two zones in North and Murrells Inlets (Fig. 2). The high-marsh prevalence of *L. irrorata* biomass is also not anticipated, given the snail's potentially obligatory association with *S. alterniflora* (e.g., Silliman & Newell 2003) and the plant's sparse occurrence in the high marsh where *S. alterniflora* is typically replaced by other halophytes (e.g., *J. roemerianus*, *S. patens*, *D. spicata*). *Littoraria irrorata* appears to be a conspicuous resident across the entire vegetated salt marsh in SC. Results from previous studies that typically focus only on the marsh near tidal creeks (Table 2) would appreciably underestimate the total population of *L. irrorata* and its overall significance within coastal marshes.

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## LONG-TERM VARIABILITY IN SPAT COLLECTIONS OF THE BLACKLIP PEARL OYSTER (*PINCTADA MARGARITIFERA*) IN SOLOMON ISLANDS

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**ABSTRACT** Temporal variation in abundance of spat of the blacklip pearl oyster (*Pinctada margaritifera*) was determined over an eight-year period at two sites, Gizo and Noro, in the Western Province of Solomon Islands. Spat were collected by deploying shade mesh substrata at a depth of 3 m for near-sequential two-month period. Overall, spatfall at the two sites was similar, with summer maximum of 4.9 (Gizo) and 4.7 collector<sup>-1</sup> (Noro). At both sites, significantly fewer spat were collected in late winter than in summer. Abundance of spat varied among years, although there was no consistency between the two sites regarding years of highest recruitment. Maximum settlement of spat at Gizo was 10.2 collector<sup>-1</sup> in January 2002, whereas at Noro it was 19.6 collector<sup>-1</sup> in March 2000. Mean size of spat did not vary over time, but spat at Gizo were significantly smaller (geometric mean 7.6 mm) than those at Noro (mean 8.4 mm). Predators of spat settled on the collectors throughout the year; there were significantly more gastropod predators at Noro than at Gizo. Gizo and Noro in the Western Province of Solomon Islands are reliable places for collection of blacklip pearl oyster spat. Spat can be caught all year round, although collections will be most effective between November and March.

**KEY WORDS:** blacklip pearl oyster, spat collection, Solomon Islands, temporal variability.

### INTRODUCTION

Blacklip pearl oysters (*Pinctada margaritifera*) are an economically important species in the tropical Indo-Pacific, primarily through the use of mature oysters for the culture of “black” pearls. At present, culture of black pearls in the Pacific is confined mostly to the lagoons of eastern Polynesia (Sarver & Sims 1996). In these locations, planktonic larval stages are entrained by currents within atoll lagoon systems. Expansion of the pearl industry to other parts of the region, where such lagoons are not present, requires identification of an alternative strategy for spat collection, and/or hatchery production. Friedman and Bell (1996) and Friedman et al. (1998) found that spat could be collected cheaply and easily using simple mesh bag collectors deployed on the seaward slopes of coastal coral reefs in Solomon Islands. Friedman and Bell (1999, 2000) then identified the optimal locations, site characteristics and deployment times for spat collection in Solomon Islands, and Friedman and Southgate (1999) demonstrated that the spat could be grown successfully to adult size.

A shortcoming of the studies (see earlier) on the availability of spat in Solomon Islands, and other locations in the region (Beer & Southgate 2000), is that they are short term. This poses a potential problem for investors who might consider establishing black pearl farms in the western Pacific, based on collection of wild spat. Ideally, they need a long time-series of data on abundance of spat to gauge whether sufficient oysters can be collected each year to support commercial operations.

In this study, we address this information gap for two locations in the Western Province of Solomon Islands by documenting temporal variability in abundance of blacklip pearl oyster spat on collectors deployed over an eight-year period.

### METHODS

The two sites used to assess long-term variation in abundance of blacklip pearl oyster spat in this study, Noro and Gizo (Fig. 1),

were selected because they previously yielded above average rates of spat collection in Solomon Islands (Friedman et al. 1998, Friedman & Bell 2000).

The methods used to collect spat were based on those developed for Solomon Islands by Friedman et al. (1998) and Friedman and Bell (1999, 2000). At each site, 10–14 spat collectors were attached at intervals along a 100-m longline made of 12 mm polypropylene rope. Each collector comprised a 0.4 m<sup>2</sup> panel of 55% shade mesh, folded concertina-fashion, threaded and tied in a bundle with monofilament line. Anchor lines and subsurface buoys, at 20-m intervals, were used to hold the longline at a depth of 3 m. The shade mesh spat collectors were deployed every two months, for duration of two months, each year between 1997 and 2005. Deployment and retrieval of collectors at the two sites was staggered over a few days, for logistic reasons. At both sites, spat were harvested from collectors as close as possible to the last days of December, February, April, June, August and October. These dates are hereafter referred to as periods 1–6 respectively. Exceptions to this, when collections were not made, were at Noro in December 1998, and at both sites in August 2001 and December 2004.

At harvest, the size, as dorso-ventral measurement (DVM), of each live and dead spat on each collector was recorded to the nearest mm. The numbers of the two most common predators of blacklip pearl oyster spat, crabs and gastropods (*Cymatium* spp.), were also recorded each time a collector was removed from the water.

### Data Analysis

The spat collector catch data were examined for variation within and between years' effects using a series of targeted analyses. For most analyses, we reduced the data from each period to average catch per collector; this accommodated the four samples where number of collectors per longline was other than 10.

Firstly, we used a 2-way ANOVA to compare differences in spat abundance, grouping by collection period and by site. ANOVA was carried out using Statistica for Windows 5.1 (Statsoft

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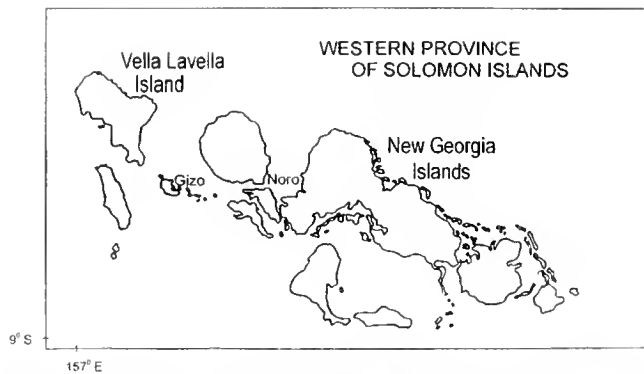


Figure 1. Location of the two sites, Gizo and Noro, in the Western Province of Solomon Islands where spat collectors were deployed in this study.

Inc., Tulsa, Oklahoma, USA). All data were transformed using  $\ln(N + 1)$  but, because of the large number of zeros, the assumption of homogeneity of variance was not met. Although ANOVA is reasonably robust to deviation from this assumption (Zar 1996), we considered differences to be significant only if  $P \leq 0.01$ . This procedure reduces the risk of Type I errors and is particularly appropriate when sample sizes are small (Underwood 1981). Significant differences between means were identified using posthoc testing (Tukey HSD test for unequal N).

We analyzed for a monotonic, long-term trend over the entire eight-year period using simple linear regression of time versus catch (Esterby 1996). We did not consider that the dataset was long enough to examine for cyclic periodicity. Where a consistent collection period effect was identified, see earlier, data were de-seasonalized before this long-term regression analysis. In practice, this was only the case for live spat numbers, and this was accomplished by calculating deviation from the overall mean value for each sampling period.

Year-to-year variation in live spat abundance was examined by comparing catches using 1-way ANOVA. For each year where all six collection periods were available, we selected the annual maximum to overcome any effects of variation in timing of peak abundance. For this analysis, spat numbers for each of the 10 replicate collectors on each line were used after transformation, using  $\ln(N + 1)$ , to improve homogeneity of variances. Insufficient numbers and multiple zero values precluded such analysis of abundances of dead spat and predators.

Differences in spat sizes among collection periods were examined using a 2-way ANOVA (site  $\times$  period). Data were skewed towards small size, which was corrected by log-transformation for all tests.

## RESULTS AND DISCUSSION

The long-term patterns of spat collection at the two sites showed variability in the abundance of spat and predators over time, and by site (Fig. 2). The way in which this variation was partitioned within years, between sites and between years, is outlined (see later).

### Within-year and Between Site Variation

When the datasets were grouped by site and collection period, no differences were found between sites, but an effect of period was evident (Table 1). The lack of an interaction effect suggests

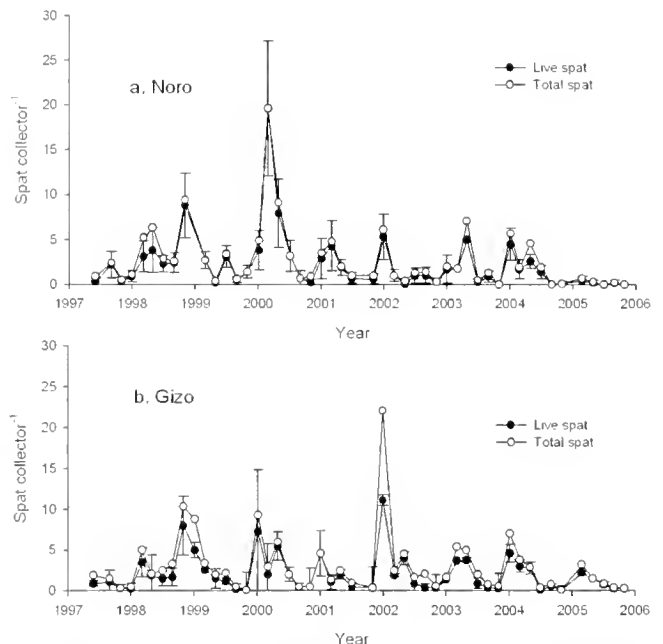


Figure 2. Mean abundance of spat (total and live) on collectors at (a) Noro and (b) Gizo in the Western Province of Solomon Islands. For each data point,  $n = 9\text{--}14$  (median  $n = 10$ ). Error bars are 95% confidence limits around live spat.

that the two sites were behaving similarly. Posthoc testing showed that the significant variation in numbers of spat collected between periods was caused by higher abundances in periods 1, 2 and 3 compared with 5 and 6, with period 4 having intermediate values. This translates to higher spatfall on collectors submerged between November and April than on those in the water from July through October. Grouped by harvest time, the lowest mean spat abundance per collector over the eight-year period for Gizo was 0.7 (June–August deployments) and the highest was 4.9 (November–December deployments). For the same approximate periods at Noro, winter yielded 1.4 spat per collector, whereas summer yielded 4.7 spat per collector. Abundances of dead spat did not differ significantly between sites but there was a significant effect of deployment period (Table 2). Once again, posthoc testing showed that collection rates were significantly lower in winter compared with summer. The pattern of a summer maximum in abundance of spat over the eight-year period was similar to that reported for the shorter study by Friedman et al. (1998). It is

TABLE 1.

Result of a 2-way ANOVA of number and size of *P. margaritifera* spat on collectors grouped by collection period (1–6) and site (Noro or Gizo). Numbers of spat were transformed using  $\ln(N + 1)$ . Significant effects ( $P < 0.01$ ) are italicized.

Variable	Source of Variation	DF	MS	F	P
Number	Site	1	0.01	0.01	0.88
	Period	5	2.77	8.57	<0.001
	Site $\times$ Period	5	0.05	0.17	0.97
	Residual	82	0.32		
Size	Site	1	6.97	17.7	<0.001
	Period	5	4.43	11.3	<0.001
	Site $\times$ Period	5	3.34	8.8	<0.001
	Residual	1794	0.39		

TABLE 2.

Results of the 2-way ANOVA of effects of deployment period and site on the number of dead *P. margaritifera*, and numbers of crabs and *Cymatium* spp. on spat collectors at Gizo and Noro. All variables were transformed using  $\ln(N + 1)$ . Effects considered significant are italicized.

Variable	Source of Variation	DF	MS	F	P
Dead spat	Site	1	0.72	4.81	0.03
	Period	5	0.55	3.70	0.004
	Site $\times$ Period	5	0.29	2.94	0.10
	Residual	82	0.15		
Crabs	Site	1	0.04	0.14	0.70
	Period	5	0.64	2.05	0.08
	Site $\times$ Period	5	0.73	2.33	0.04
	Residual	82	0.31		
<i>Cymatium</i>	Site	1	0.76	8.01	0.006
	Period	5	0.06	0.67	0.65
	Site $\times$ Period	5	0.12	1.25	0.29
	Residual	82	0.09		

interesting that the shorter soak time of spat collectors in our study (2 months) yielded the same peak numbers of spat as the six-month soak time used by Friedman et al. (1998). This is consistent with the view that there is an optimal soak time before predators begin to overwhelm ongoing recruitment and perhaps explains why we collected relatively few (23%) dead spat compared with Friedman et al. (1996) (46%). The timing of peak spat settlement in Solomon Islands differed to that found on the Great Barrier Reef, where maximum numbers were collected during May to June (Beer & Southgate 2000). Reasons for this difference are unclear. Crabs and *Cymatium* spp. showed no consistent variation in abundance among collection periods, although *Cymatium* spp. were more abundant at Noro (average of 0.53 collector<sup>-1</sup>) than at Gizo (0.33 collector<sup>-1</sup>) (Fig. 3, Table 2).

#### Inter-annual Variation and Multiyear Trends

Live spat showed no multiyear trends in abundance after the data were de-seasonalized. Nevertheless, there were clearly differences in abundance of spat in some years, which appear to be caused by differences in timing of the recruitment peak and in size of the peaks (Fig. 2). Out of 13 site-years when a full year of data were collected, the summer peak was in period 1 in 46% of cases, period 2 in 30%, period 3 in 15% and period 6 in 8%. At Gizo, the greatest annual spatfall peak occurred in period 6 in 2001 (10.2 collector<sup>-1</sup>) and at Noro in period 2 of 2000 (19.6 collector<sup>-1</sup>). We examined the significance of the magnitudes of annual peaks using 1-way ANOVA for each site (Table 3). Only years when data were obtained for all sampling periods could be used, and this removed 2005 from both sites. Note that for this analysis, because of one occasion when the summer peak spat collection was in period 6 of the previous calendar year, we considered that each spat year began with period 6 of the calendar year before and ran through to period 5 of the year in question. Significantly different peak spatfalls were evident between years at both sites. Posthoc testing showed that at Noro the 2000 peak yielded more spat than any other year except 1999, whereas at Gizo, abundance in 2002 was significantly higher than in 1998, 2001, 2003 and 2004, with no other significant differences.

The number of crabs at Gizo was the only temporal dataset that

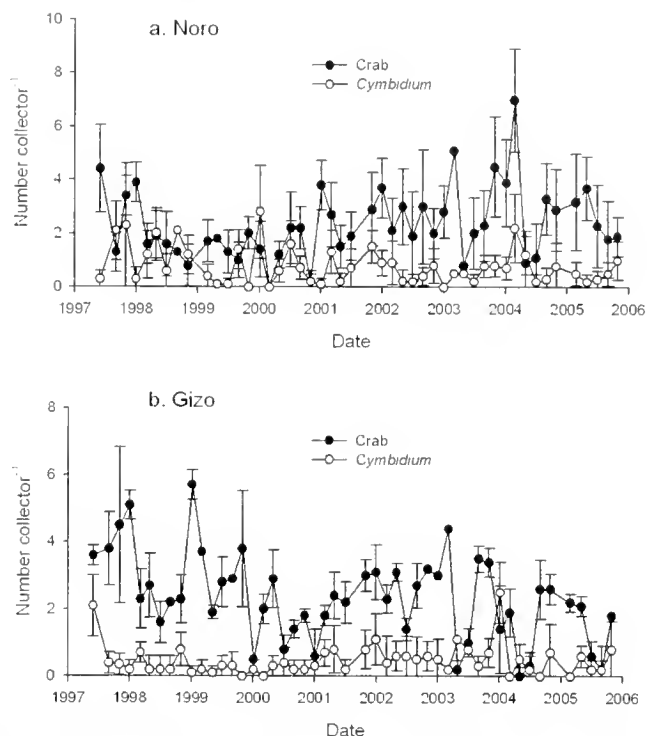


Figure 3. Mean abundance of two major predators of blacklip pearl oyster spat, crabs and *Cymatium* spp. on collectors at (a) Noro and (b) Gizo. For each data point,  $n = 9-14$  (median  $n = 10$ ). Error bars are 95% confidence limits.

showed evidence of a linear trend over time, in this case one of declining abundance (Table 4). The absence of any reciprocal effect on spat numbers shows that, under the conditions of deployment, the small changes in crab numbers did not affect spat survival.

#### Spat Size

There were significant differences in the mean sizes of spat harvested during each collection period and between sites (Table 1). Maximum size tended to be in period 2, towards the latter part of summer, when water temperatures can be expected to be highest. This effect was more pronounced at Gizo than at Noro. The median sizes of live spat on collectors at Noro and Gizo were 9 and 7 mm, and the geometric means were significantly different at 7.6 and 8.1 mm, respectively (Table 1). Caution should be taken in interpreting this size difference, because this result was obtained

TABLE 3.

Results of the 1-way ANOVA of effects of year on the maximum number of *P. margaritifera* spat ( $\ln(N + 1)$ ) on spat collectors deployed at any period within that year at Noro and Gizo. Only years when data from 10 individual spat collectors were available for each of the six sampling periods were used in this analysis.

Site	Source of Variation	DF	MS	F	P
Noro	Year	6	2.84	6.77	<0.001
	Residual	61	0.42		
Gizo	Year	6	1.63	3.49	0.005
	Residual	62	0.46		

TABLE 4.

Results of the linear regression analysis of the time-series from Gizo and Noro of mean numbers of live spat (de-seasonalized), crabs and *Cymatium* spp. Regression slopes (as change in catch collector<sup>-1</sup> y<sup>-1</sup>) are given only for significant regressions.

Site	Variable	R <sup>2</sup>	F	df	p	Slope ± error
Noro	Live Spat (de-seasonalized)	0.06	3.42	1,47	0.07	
	Crabs	0.06	2.53	1,47	0.12	
	<i>Cymatium</i> spp	0.06	2.44	1,47	0.12	
Gizo	Live Spat (de-seasonalized)	0.03	1.17	1,47	0.28	
	Crabs	0.16	10.3	1,47	<0.01	-0.21 ± 0.07
	<i>Cymatium</i> spp	0.00	0.11	1,47	0.74	

from a large dataset, and the differences were within the likely measurement error of spat. Thus, the ecological significance of such a small difference is questionable. The arithmetic mean of spat for both sites was 10 mm. This is consistent with the earlier data of Friedman and Bell (2000), who reported that spat attained

an arithmetic mean size of ~10–14 mm within three months of deployment of collectors.

## CONCLUSION

Gizo and Noro in the Western Province of Solomon Islands are reliable places for the collection of blacklip pearl oyster spat. However, collection of spat is expected to be most cost-effective between November and March, when average yields of >4 spat per collector for two-month deployments can be expected in most years.

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## THE LIMITS OF MORPHOMETRIC FEATURES FOR THE IDENTIFICATION OF BLACK-LIP PEARL OYSTER (*PINCTADA MARGARITIFERA*) LARVAE

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**ABSTRACT** As with most cultivated bivalves, culture of the Tahitian pearl oyster *Pinctada margaritifera* is particularly dependent on the natural environment, especially for spat supply. The ability to track in real time the abundance and the development of pearl oyster larvae in the plankton would help optimize spat collection in atolls of French Polynesia. However no identification criteria are available for the larvae of several bivalves species present in the lagoons and it is not yet possible to specifically monitor pearl oyster larvae. The aim of this study is to determine the most pertinent morphological identification criteria, to specifically identify the larvae of *P. margaritifera* and differentiate them from those of three other abundant species: *Pinctada maculata*, *Crassostrea cuculata* and *Chama* sp. The method of image analysis after photon microscopy was assessed. It allowed automatic measurement of numerous morphometric features that were tested alone or in combination and identification threshold for *P. margaritifera* larvae were determined by statistical analyses. These results led to a key that allowed correct identification for 77% of *P. margaritifera* larvae. The hinge diagnosis method under scanning electron microscopy, a prime method for the identification of specific criteria on bivalve larva shells, was also used on larvae of both *Pinctada* species. The two species could be differentiated precisely because of specific differences in the thickness of their hinge proinaculum and the number of denticles it bears. However this approach is too time-consuming and technically demanding to use in real time field studies. This study showed the limitations of image analysis as an identification tool of the *P. margaritifera* larvae, but proper statistical analyses and especially the decision tree approach could be used to evaluate and efficiently prioritize the choice of the species identification criteria.

**KEY WORDS:** *Pinctada margaritifera*, larval identification, morphological features

### INTRODUCTION

The culture of the pearl oyster *Pinctada margaritifera* (Linné, 1758) is a recent activity in French Polynesia. It developed rapidly in the 1980s and today represents the second source of income after tourism. Production reached its peak in 2000 with more than 11 tons of raw pearls with a value of 168 million Euros and generated 7,000 jobs in more than 1,000 farms spread out over some 30 islands. Subsequently, the pearl industry faced a crisis caused by a steep drop in pearl prices, which started in 2001. Export values were 122 million Euros in 2002 and 84 million Euros in 2003 for tonnages of 11 tons and 10 tons respectively. This was mostly because of a very swift and poorly controlled increase in production that entailed a perceptible drop in quality. This crisis highlighted the necessity to organize the market, and also to regulate and to sustain production.

To a large extent, the culture of the *Pinctada margaritifera* oyster in French Polynesia depends on the natural environment and it relies entirely on the supply of collected spat. The professionals set their collectors up in an empirical way based on their past observations, but spat yields show a large spatio-temporal variability (Cabral et al. 1985, Brié, pers. comm., 1999). The ability to follow-up the larvae during their pelagic life could be useful to predict the periods and places of post larvae settlement and would thus help to improve spat collection as well as spat quality. Tools are therefore needed for the identification of pearl oyster larvae in plankton samples. To our knowledge, no attempt has been made so far to identify any of the bivalve larvae present in the waters of French Polynesian atolls. Techniques allowing larvae of *P. margaritifera* to be recognized among the larvae of other bivalves

must be developed. Such approaches should, in particular, allow the distinction between larvae of *Pinctada margaritifera* and of *P. maculata* (Gould, 1850), which could be a competitor for settlement sites.

The identification of sampled larvae is always difficult and a review of techniques currently available is given in Garland and Zimmer (2002). Techniques of image analysis are explored here as a first step towards the field identification of pearl oyster larvae. The aim of this study is to evaluate the main morphological descriptors of the larvae of 4 common species in the plankton samples from Polynesian pearl farming atolls: *P. margaritifera*, *P. maculata*, *Crassostrea cuculata* and *Chama* sp. (Broderip, 1834).

Image analysis was retained as a working method to increase the number of descriptive variables of the study. Automated analysis on computer would also greatly help in reducing the subjectivity of optical methods. Univariate and multivariate statistical treatments were then applied. These methods identified the most pertinent morphological characteristics for identification of these 4 species of bivalves.

### MATERIAL AND METHODS

#### *Larval Samples From the Hatchery*

The larvae of four main bivalve species present in the Polynesian atolls were reared in hatchery up to metamorphosis. *Chama* sp., *Crassostrea cuculata* and *Pinctada margaritifera* larvae were produced at the Service de la Perliculture in Rangiroa. *Pinctada maculata* and *Pinctada margaritifera* larvae were produced at Ifremer, Center du Pacifique in Tahiti.

Samples were taken on a regular basis in rearing tanks to obtain larvae of various ages ranging from 1–24 days (Table 1).

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TABLE 1.  
Species and age of analyzed larvae.

Stage (aver.)	Age in Days	<i>Chama</i> sp.	<i>C. cuculata</i>	<i>P. maculata</i>	<i>P. margaritifera</i>
		Number	Number	Number	Number
D	1	5	1		7
	2		24		
	3				44
	4	12	7		
	5				98
	6	14	10	9	
	7				104
U	8		13		
	9				85
	10	33	5	70	
	11			72	128
	13	18	2	94	160
	15				38
	16			78	
P	17	36	2		49
	19	28			222
	20			106	
	21				73
	22	27	26	76	
	23				72
	24			11	
	Total	173	90	516	1080

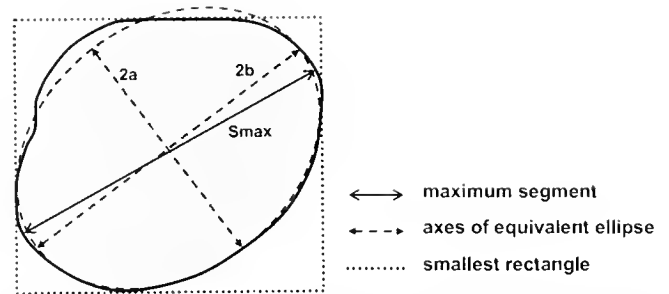


Figure 1. Representation on a larva contour of the dimensions used for calculating 3 ( $S_{max}$ ,  $2a/2b$ , Comp) of the 6 parameters retained for the morphometric analysis.

#### Morphological Observations

Larvae were observed with a LEITZ DMRE photon microscope. A RGB mode (Red, Green, Blue) digitized picture was acquired for each of the larvae and was analyzed with Optilab software.

Each of the RGB pictures was separated into 3 distinct layers, green, red and blue. Only the green layer was retained, because it presented the highest saturation levels and it was converted into the 256 gray level mode. The areas representing larvae were those having the highest values of gray. The automatic selection of all the pixels was done depending on their value of gray and the smallest value of gray corresponding to the bivalve larvae was empirically determined for each picture. This value was retained as

TABLE 2.  
Mean and SE for each variable measured for each stage of each species.

		D		U		P	
Species/Stage		Mean	SD	Mean	SD	Mean	SD
Smax	<i>P. margaritifera</i>	157.03	61.37	168.94	76.45	206.91	72.07
	<i>P. maculata</i>	134.95	25.15	136.42	31.88	208.73	21.75
	<i>Chama</i> sp.	173.15	42.45	413.66	19.47	159.58	65.56
	<i>C. cuculata</i>	184.37	38.40	276.37	57.78	382.26	35.86
2a/2b	<i>P. margaritifera</i>	1.298	0.151	1.255	0.131	1.286	0.145
	<i>P. maculata</i>	1.389	0.155	1.312	0.159	1.347	0.148
	<i>Chama</i> sp.	1.205	0.119	1.307	0.142	1.297	0.151
	<i>C. cuculata</i>	1.228	0.102	1.197	0.144	1.272	0.108
Elong	<i>P. margaritifera</i>	1.414	0.089	1.401	0.078	1.416	0.087
	<i>P. maculata</i>	1.492	0.096	1.428	0.097	1.394	0.079
	<i>Chama</i> sp.	1.354	0.055	1.379	0.053	1.412	0.088
	<i>C. cuculata</i>	1.380	0.062	1.389	0.067	1.383	0.063
Comp	<i>P. margaritifera</i>	0.769	0.025	0.772	0.021	0.765	0.026
	<i>P. maculata</i>	0.761	0.029	0.767	0.026	0.767	0.029
	<i>Chama</i> sp.	0.775	0.018	0.767	0.024	0.769	0.025
	<i>C. cuculata</i>	0.777	0.019	0.773	0.018	0.768	0.028
Ftype	<i>P. margaritifera</i>	0.992	0.007	0.994	0.004	0.991	0.006
	<i>P. maculata</i>	0.984	0.011	0.990	0.009	0.991	0.004
	<i>Chama</i> sp.	0.995	0.004	0.993	0.004	0.992	0.007
	<i>C. cuculata</i>	0.996	0.004	0.995	0.006	0.994	0.002
Ixy	<i>P. margaritifera</i>	4.471E + 05	1.247E + 07	7.479E + 05	1.812E + 07	1.218E + 06	1.987E + 07
	<i>P. maculata</i>	6.031E + 04	2.503E + 06	-3.451E + 06	2.023E + 06	1.818E + 05	5.954 + 06
	<i>Chama</i> sp.	-1.668E + 06	3.936E + 06	-1.524E + 07	7.593E + 07	3.555E + 04	1.508E + 07
	<i>C. cuculata</i>	1.998E + 05	5.548E + 06	-1.402E + 07	5.360E + 07	8.762E + 06	4.884E + 07

D: D larva; U: umbo larva; P: pediveliger.

TABLE 3.  
Comparison of 6 variable average values for each larval stage and each species at 5% level.

	Species/Stage	Test	P	Sign.	Compl. Test
Smax					
Effect of stage	<i>P. margaritifera</i>	K & W	0.002	S	D > U > P
	<i>P. maculata</i>	K & W	0.002	S	D > U > P
	<i>Chama</i> sp.	K & W	0.008	S	D > U > P
	<i>C. cuculata</i>	K & W	0.002	S	D > U > P
Effect of species	<i>D</i>	ANOVA	<0.0001	S	m = M < C = K
	<i>U</i>	K & W	0.009	S	m = M < C = K
	<i>P</i>	ANOVA	0.006	S	m = M < C = K
2a/2b					
Effect of stage	<i>P. margaritifera</i>	ANOVA	0.890	NS	
	<i>P. maculata</i>	K & W	0.053	NS	
	<i>Chama</i> sp.	ANOVA	0.430	NS	
	<i>C. cuculata</i>	ANOVA	0.285	NS	
Effect of species	<i>D</i>	K & W	0.236	NS	
	<i>U</i>	ANOVA	0.811	NS	
	<i>P</i>	ANOVA	0.972	NS	
Elong					
Effect of stage	<i>P. margaritifera</i>	K & W	0.356	NS	
	<i>P. maculata</i>	ANOVA	0.289	NS	
	<i>Chama</i> sp.	ANOVA	0.328	NS	
	<i>C. cuculata</i>	ANOVA	0.766	NS	
Effect of species	<i>D</i>	ANOVA	0.534	NS	
	<i>U</i>	ANOVA	0.264	NS	
	<i>P</i>	K & W	0.764	NS	
Comp					
Effect of stage	<i>P. margaritifera</i>	ANOVA	0.684	NS	
	<i>P. maculata</i>	K & W	0.180	NS	
	<i>Chama</i> sp.	ANOVA	0.032	S	D = U > P
	<i>C. cuculata</i>	K & W	0.150	NS	
Effect of species	<i>D</i>	ANOVA	0.599	NS	
	<i>U</i>	K & W	0.440	NS	
	<i>P</i>	ANOVA	0.872	NS	
Ftype					
Effect of stage	<i>P. margaritifera</i>	K & W	0.327	NS	
	<i>P. maculata</i>	K & W	0.134	NS	
	<i>Chama</i> sp.	ANOVA	0.758	NS	
	<i>C. cuculata</i>	K & W	0.031*	S	U > D = P
Effect of species	<i>D</i>	ANOVA	0.567	NS	
	<i>U</i>	K & W	0.174	NS	
	<i>P</i>	ANOVA	0.362	NS	
Ixy					
Effect of stage	<i>P. margaritifera</i>	K & W	0.108	NS	
	<i>P. maculata</i>	K & W	0.470	NS	
	<i>Chama</i> sp.	K & W	0.008	S	D = U > P
	<i>C. cuculata</i>	K & W	0.810	NS	
Effect of species	<i>D</i>	K & W	0.234	NS	
	<i>U</i>	K & W	0.018	S	m = M < C = K
	<i>P</i>	K & W	0.390	NS	

NS, Not significant; S, significant; D, *D* larva; U, umbo larva; P, pediveliger; M, *P. margaritifera*; m, *P. maculata*; C, *Chama*; K, *C. cuculata*; K & W, Kruskal & Wallis; \*, before Bonferroni correction.

a threshold to binarize the picture. The value 1 (corresponding to the larvae) was assigned to areas with gray value greater than the threshold, and the value 0 was assigned to areas with gray value lower than the threshold. Areas of value 1 that were not complete were filled in automatically and among them, only those corresponding to individualized larvae were retained and colored in red.

The Optilab software automatically numbers the retained red colored areas and describes them by performing a series of 42 measurements such as coordinates, optical density, dimensions and

shape parameters. The best descriptors among this set of 42 were determined using principal component analysis (PCA). Six descriptors, the least dependent, were retained to establish a morphometric analysis pattern that would result in the specific identification of the 4 species of bivalves studied. They constituted the descriptive variables of larvae morphology (Fig. 1):

- the maximum intercept (Smax): length of the longest segment of the object.

TABLE 4.

Correlation matrix of the 6 least correlated parameters selected after a PCA

	Smax	2a/2b	Elong	Comp	Ftype	Ixy
Smax	1					
2a/2b	0.039	1				
Elong	0.047	0.259	1			
Comp	-0.127	-0.113	-0.524	1		
Ftype	-0.053	-0.419	-0.223	0.324	1	
Ixy	-0.035	-0.003	-0.014	-0.016	0.010	1

None of these correlations are significant at the 5% level after Bonferroni correction.

- the Ellipse Ratio (2a/2b): ratio of the major axis to the minor axis of the equivalent ellipse.
- the Elongation Factor (Elong): ratio between Smax and the mean perpendicular intercept.
- the Compactness Factor (Comp):  $A/A_R$  where A is the object area and  $A_R$  is the area of the smallest rectangle containing the object.
- the moment of inertia (Ixy) representing the pixel distribution around the Center of Mass xy:  $(\sum xy) - A \cdot Mx \cdot My$ , where xy are the pixel coordinates, Mx is the Center of Mass x  $[(\sum x)/A]$  and My is the Center of Mass y  $[(\sum y)/A]$ .
- the Type Factor (Ftype):  $A^2/4 \cdot \pi \sqrt{Ixx \cdot Iyy}$ , where Ixx is the Inertia xx  $[(\sum x^2) - A \cdot Mx^2]$  and Iyy is the Inertia yy  $[(\sum y^2) - A \cdot My^2]$ .

#### Data Analysis

##### Univariate Analysis

The morphometric measurements of 4 species were classified into 3 subgroups corresponding to the main stages of physiological

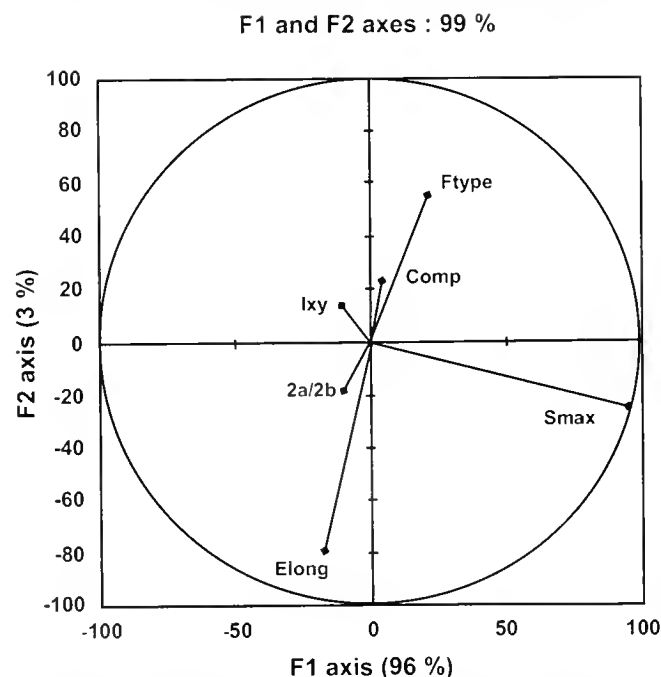


Figure 2. Correlation circle for F1 and F2 axes of the DFA applied to 6 measurements. Most of the interspecific variability (96%) is represented by Smax on F1 axis.

Individuals (F1 and F2 axes: 99 %)

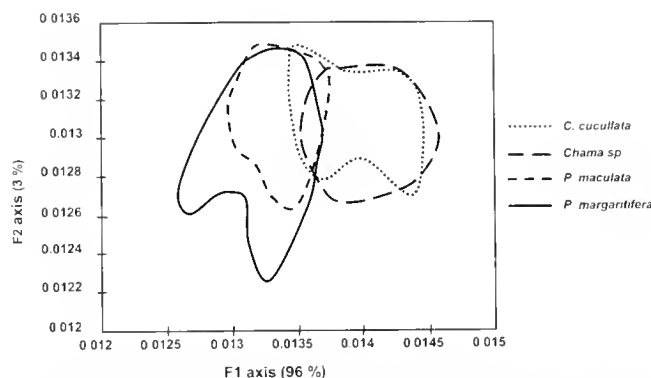


Figure 3. Situation of species patches in the F1-F2 canonical space. Patches are designed to contain approximately 95% of species dots. The best species discrimination, along F1 axis, separates the genus *Pinctada* on the left from *Chama* sp and *Crassostrea cucullata* on the right.

and morphological development: D larva (D), umbo larva (U) and pediveliger larva (P). An analysis of variance (ANOVA or test of Kruskal & Wallis) was undertaken for each of the 6 descriptive variables according to the species and the larval stage.

#### Discriminant Factor Analysis

The discriminant factor analysis (DFA) was carried out with the help of Excelstat 5.1 software. The 6 descriptors retained to specifically discriminate the larvae were not significantly correlated (the highest  $r$  was  $-0.524$  between Elong and Comp). It was then possible to use a DFA approach to search for the combination of descriptors that maximized interspecific variability and minimized intraspecific variability (regardless of the larval stage encountered).

#### Decision Tree

This analysis, realized with Statistica 6 software, complemented DFA to identify larvae of the 4 species from the batches of hatchery monospecific larvae. Starting with all individuals attributed to the same species, it supplied a true-false determination model, with intermediate nodes of decision and terminal nodes of species attribution. The analysis stopped when the original species was found again with an error margin defined by the conditions for halting the segmentation, set here in advance to a minimum of 5 successive levels.

#### Electron Microscopy

About 10 hatchery larvae of each *P. margaritifera* and *P. maculata* species were prepared for SEM observation. Larvae were cleaned from organic matter in a bath of 10% chlorine water (Le Pennec 1978). This treatment was controlled under the microscope and was stopped by washing with distilled water as soon as degassing was observed within the valves. The prodissoconch valves were carefully separated using mounted pins and were dehydrated in a bath of 90% alcohol. They were then set up on plots for metal plating. The valves thus prepared were observed under a HITACHI-S 3200 N scanning electron microscope.

TABLE 5.  
Specified identification success rate of individual larvae (all stage combined) by DFA.

True species	<i>C. cuculata</i>	<i>Chama</i> sp.	<i>P. maculata</i>	<i>P. margaritifera</i>
Individuals	89	168	523	1,077
Sorting				
<i>C. cuculata</i>	23	28	33	57
<i>Chama</i> sp.	30	117	0	0
<i>P. maculata</i>	23	17	359	375
<i>P. margaritifera</i>	13	6	131	645
Correct identification	26%	70%	69%	60%

## RESULTS

### Univariate Analyses

The mean values of the 6 descriptive variables of larvae morphology (Table 2) were compared by a one-way variance analysis or a Kruskal & Wallis test (Table 3). Overall, there was no significant difference at the threshold of 5%, between means of the different groups during the larval development, except for:

- Ftype of the *C. cuculata* U stage was significantly higher than that of the D and the P stages. This was not significant however after Bonferroni correction;
- Comp of *Chama* sp was similar for the D to U larval phase but significantly smaller at the P stage; this was not significant anymore after Bonferroni correction.
- Ixy of the *Chama* species at the P stage was significantly different from that of the D or U stages even after Bonferroni correction. It also allowed to differentiate this species from the other three for the stage U, but this was not significant after Bonferroni correction;
- Smax allowed distinguishing the three main stages of larval development regardless of the species, because all comparisons within species between stage were significant even after Bonferroni correction. All comparisons between species within stage were also significant even after Bonferroni correction and Smax was especially useful to significantly differentiate the group of individuals composed of *P. margaritifera* and of *P. maculata* from the group of individuals composed by *Chama* sp and *C. cuculata*.

None of the six morphometric measurements allowed to discriminate alone the larvae of the four species. It was at best pos-

sible to distinguish individuals of *Chama* sp or of *Crassostrea cuculata* from those of *P. margaritifera* and of *P. maculata*.

### Discriminant Factor Analysis

The principal component analysis (PCA) carried out on the 42 morphometric measurements allowed to retain among them the 6 least correlated parameters (Table 4).

The DFA results showed that the two first canonic axes F1 and F2 respectively represented 96% and 3% of the interspecific variability (Fig. 2). Smax corresponded mainly to F1 axis whereas Elong, in opposition to Ftype, corresponded more to F2 axis. The dot patches of the 4 species individuals are shown in the two dimensions canonical space set by axes F1 and F2 (Fig. 3). Groups of both *Pinctada* species individuals were clearly superimposed. Smax (mainly represented by F1 axis) allowed their discrimination, regardless of the larval stage, from the groups of *Chama* and *Crassostrea cuculata* species, superimposed as well. Further differentiation between the individuals of both species of *Pinctada* or between the *Chama* or *C. cuculata* species was not possible along F2 axis.

A Kullback statistical test revealed that the difference between the intragroup variance-covariance matrices was significant at 5% ( $X^2 = 2340.3$ ;  $P < 0.0001$ ). The barycenters corresponding to the morphometry of the 4 studied species were significantly different (Wilks  $\Lambda$  ratio = 0.433;  $F = 99.88$ ;  $P < 0.0001$ ). A DFA was carried out based on these characteristic barycenters to try species identification of each larva previously measured, but the retained discriminant morphometric features were not sufficient. The identification success rates varied depending on the species (Table 5). They were comprised between 60% and 70% for both *Pinctada* species and the *Chama* genus, whereas only 26% was achieved for the *Crassostrea* genus.

TABLE 6.  
Species identification success rate of D individual larvae by DFA.

True species	<i>C. cuculata</i>	<i>Chama</i> sp.	<i>P. maculata</i>	<i>P. margaritifera</i>
Individuals	21	5	6	70
Sorting				
<i>C. cuculata</i>	15	0	0	0
<i>Chama</i> sp.	5	5	0	0
<i>P. maculata</i>	0	0	5	21
<i>P. margaritifera</i>	1	0	1	49
Correct identification	71%	100%	83%	70%

TABLE 7.  
Species identification success rate of U individual larvae by DFA.

True species	<i>C. cuculata</i>	<i>Chama</i> sp.	<i>P. maculata</i>	<i>P. margaritifera</i>
Individuals	15	14	87	77
Sorting				
<i>C. cuculata</i>	10	3	2	1
<i>Chama</i> sp.	4	8	1	0
<i>P. maculata</i>	1	1	58	31
<i>P. margaritifera</i>	0	2	26	45
Correct identification	67%	57%	67%	58%

Other DFA (Tables 6, 7 and 8) were undertaken on these samples to see if better species identification could be achieved at specific larval stages with the morphometric measures at hand. The correct identification rates were quite different from one species to another, but also between larval stages. The D and P stages gave the best species identification success rates overall.

#### The Decision Tree

A decision tree was built to help synthesize the results of the various DFA. Each morphometric measurement was assimilated to a prediction variable (specific identification criteria). After univariate preliminary studies, they were classified according to their potential (0–100 scale) to predict the dependent variable (species identity). Ftype, Smax, Elong and the larval stage had a potential of 100, 96, 72 and 59 respectively to explain the dependent variable.

The organization of the tree was performed arbitrarily in the following manner: All of the larvae were allocated at first to the *P. maculata* species. If Smax was larger than 271  $\mu\text{m}$ , the larva identity was changed for a *Chama* larva. If this condition was not true, the larva was still assigned to the *P. maculata* species until the next decision node. The overall process of the decision tree (Fig. 4) led to a discrimination key. Synthetic results of the larval identification using this discrimination key were better than those obtained with the former DFA (Table 9). However, species identification error rates were still generally higher than 25%.

#### Hinge Analysis by Electron Microscopy

The hinge of the umbo larva of *P. margaritifera* is made up of a narrow provinculum bearing 4 denticles on the anterior edge and 6 on the posterior edge (Fig. 5-1). The hinge of the umbo larva of *P. maculata* has a thick provinculum bearing 5 denticles on the anterior edge and 5 on the posterior edge (Fig. 5-2). There

was no individual variation within species for these traits, thus hinge analysis of larval shells allowed the formal distinction between both species of the *Pinctada* genus within the larval phase.

#### DISCUSSION

Thirty-four species of bivalves were found in the lagoon of the atoll of Takapoto (Salvat & Richard 1985). All of them are liable to produce larvae at the same time as the black lip pearl oyster. Therefore identification and monitoring of *P. margaritifera* larvae is not possible without means to discriminate this bivalve species from others present in the lagoon. The objective of this study was to test different morphological descriptors and to check whether the most pertinent among them could be used as tools for species determination.

The analysis of the shell profile using shape descriptors is a technique commonly used to determine numerous plankton species and was tested here. It was then related to the analysis of the hinge structure (ornamentation descriptors), a permanent criteria allowing a definite species determination (Le Pennec 1978).

Image analysis applied to morphometry has proven efficient in taxonomic identification (Garland & Zimmer 2002). This method presents the advantage of replacing the observer by algorithms and thus minimizing the risk of subjective errors. It is fast and efficient for numerous applications, such as the study of zooplankton communities. (Beaulieu et al. 1999). The data generated are useful in solving taxonomy questions and sometimes even to identify morphological variation among populations within a species (Jeffries et al. 1984, Ishii et al. 1987, Cadrin & Friedland 1999). Whereas morphometric analysis is successful at species identification in some cases, it is more difficult for a number of others. This is the case of a few copepods and fish larvae as described by Naudin et

TABLE 8.  
Species identification success rate of P individual larvae by DFA.

True species	<i>C. cuculata</i>	<i>Chama</i> sp.	<i>P. maculata</i>	<i>P. margaritifera</i>
Individuals	46	152	423	316
Sorting				
<i>C. cuculata</i>	21	38	1	2
<i>Chama</i> sp.	19	91	0	0
<i>P. maculata</i>	4	16	312	108
<i>P. margaritifera</i>	2	7	110	206
Correct identification	46%	60%	74%	65%

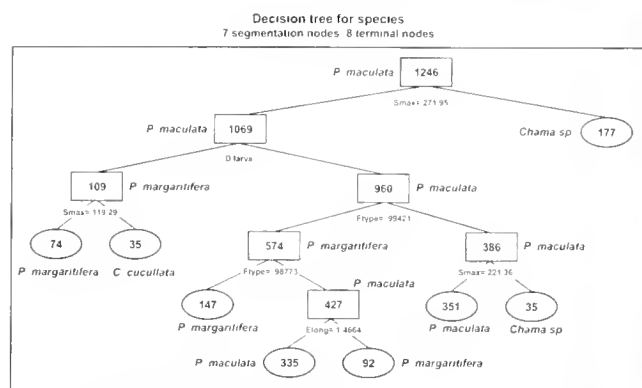


Figure 4. Decision tree for discrimination of the four species. Each rectangle box represents a segmentation node under which value of the discrimination factor is given. Each ellipse box represents a terminal node of species determination. Numbers given in the node boxes represent larvae counts.

al. (1996). The transparency of the objects to analyze is a widely encountered difficulty. In the case of bivalve larvae, the shells are translucent but their profiles are generally sufficiently contrasted from the rest of the picture and correspond to closed forms. It is then possible to use data processing tools to analyze each picture, cut out all closed forms and describe them according to contour, area and position in the picture.

The measurements performed on all of the larval stages of the 4 bivalve species studied here provided information on some of their distinctive dimensions according to age.

Univariate analyses showed that Smax was the most discriminating measure between stage within species and between species within stage. The multivariate analyses gave better results. Some degree of species identification among the larvae of the 4 species was possible, but the rates of success were different according to the methods used:

- Using DFA, the larvae discrimination of *P. margaritifera*, of *P. maculata*, of *Chama* and of *C. cucullata* was achieved with probabilities of success of 60%, 69%, 70% and of 26% respectively.
- With the decision tree approach, the larvae discrimination of *P. margaritifera*, of *P. maculata*, of *Chama* and of *C. cucullata* was achieved with probabilities of success of 74%, 63%, 69% and of 77% respectively.

Despite these positive results, the descriptors studied here cannot be considered adequate for the identification of *Pinctada* and

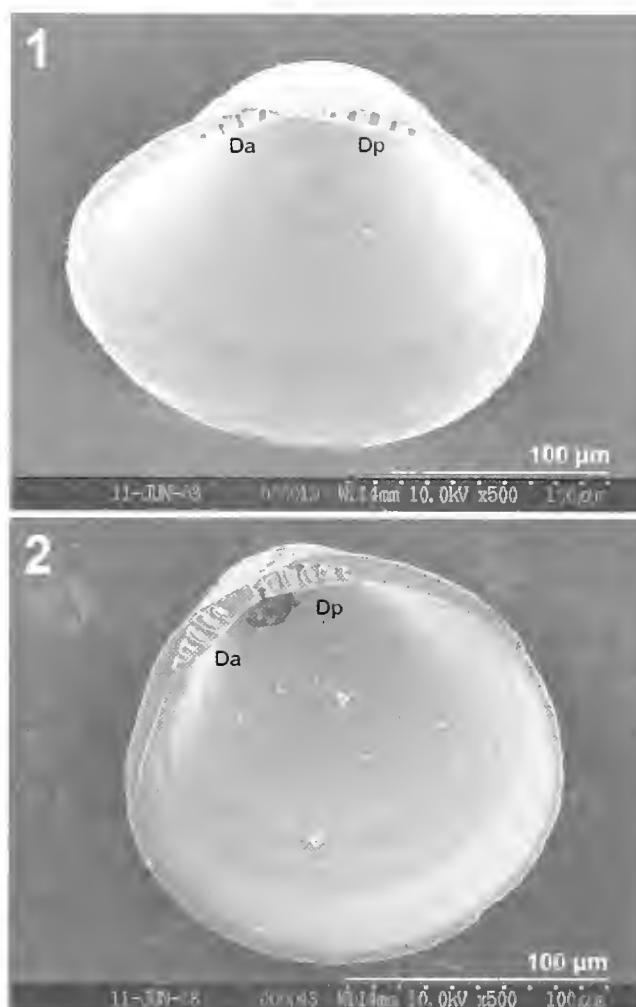


Figure 5. SEM photographs of right valve of *P. margaritifera* (1) and *P. maculata* (2) larvae. Numbers of anterior teeth (Da) and of posterior teeth (Dp) of the hinge are invariant and differ between both species.

other bivalve larvae in plankton samples. A higher reliability and a success probability close to 90% would be required, as suggested in the literature (Jeffries et al. 1984, Naudin et al. 1996, Rahhou, 2003). Similar discrimination rates (up to 74%) between bivalve larvae were obtained by Hendriks et al. (2005), who concluded that shape characteristics combined with computer analysis did not provide tools conclusively useful for young bivalve larvae identification.

TABLE 9.

Species identification success rate of P individual larvae using a decision tree.

True species	<i>C. cucullata</i>	<i>Chama</i> sp.	<i>P. maculata</i>	<i>P. margaritifera</i>
Individuals	35	212	686	313
Sorting				
<i>C. cucullata</i>	27	49	12	2
<i>Chama</i> sp.	5	147	20	1
<i>P. maculata</i>	0	5	434	77
<i>P. margaritifera</i>	3	11	220	233
Correct identification	77%	69%	63%	74%

Another problem is that the acquired image of the larva form can vary between records, according to the orientation of the shell. To avoid image distortion and measurement errors, the separation plane between valves should be parallel to the sensor plane. Accuracy may also improve with additional measurement of the valve thickness. This descriptor has already been the subject of an original application as a quality indicator of larval development for *Pecten maximus* (Salaün et al. 1991). Image multiaquisition resulting from slightly moving the observed larva or changing the angle of light reaching the larva may also help in selecting larval areas that present the largest  $S_{max}$ .

More sophisticated mathematical contour descriptors such as Fourier descriptors (Crampton 1995) could also be considered, because it is possible to decompose the contour of an object using the Fourier elliptic transform. This method has already been proposed by Gevirtz (1976) to evaluate specific and intraspecific variability amongst bivalves, and these descriptors have been successfully used to distinguish similar mussel species (Crampton 1995, Innes & Bates 1999) and to identify eel populations based on otolith shape (Rahhou 2003). Davis et al. (1996) anticipate ongoing improvement of these visualization techniques for plankton identification, but phenotypic plasticity during larval development will limit their accuracy (Garland & Zimmer 2002).

The method of valve hinge analysis proved again its discrimination potential (Le Pennec 1978) in the present study. It enabled a definite distinction of both *Pinctada* species according to the number of denticles and to the thickness of the provinculum. However, the preparation time for each larva and the necessary handling precautions remain incompatible with the goal of a fast characterization of large-scale distributions and abundances of larvae.

Considerable progress has been achieved with other techniques

of larval identification, which are not based on the morphological but on the molecular characteristics of each species. Two tracks are being explored at this time for species identification and discrimination of bivalve larvae. One relies on genetics tools (Lopez-Piñon et al. 2002, Wood et al. 2003, Hosoi et al. 2004) and the other on immunological approach (Paugam et al. 2000, Abalde et al. 2003).

The DNA markers present the advantage of being independent of age or physiological condition of the individuals. It is therefore possible to use individual adults to develop probes that are efficient on larvae. However, their use requires in most cases that the plankton sample be destroyed, and therefore they are better suited as overall indicators of presence and representation within a sample, rather than as real probes for individual marking.

The advantage of the immunological identification over genetic identification is that the antibodies find in the plankton sample the larvae against which they have been induced and specifically mark them without altering the shell structures (Paugam et al. 2003). However the induction of an efficient antibody production requires the availability of large amounts of larvae of different species that can only be obtained in the hatchery.

The analysis of the hinge characteristics is the only method available today which would ensure identification of the *P. margaritifera* pearl oyster larvae. However, this method is too cumbersome to authorize a rapid identification of the larvae and cannot be used in real time for long term systematic plankton sampling. It could, however, be used to validate any other technique of larva, species identification. Any improvement of these techniques will be useful for following larvae within the plankton and to develop our knowledge of the larval life cycle of the *P. margaritifera* pearl oyster in the lagoons of French Polynesia.

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## RELATION OF SEASONAL CHANGES IN THE MASS OF THE GONAD AND SOMATIC TISSUES OF THE ZEBRA ARK SHELL *ARCA ZEBRA* TO ENVIRONMENTAL FACTORS

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**ABSTRACT** Over the period from June 2002 to June 2003, we examined the relationship of seasonal changes in the mass of the gonad and somatic tissues to environmental factors for four size groups of the zebra ark shell *Arca zebra* at Chacopata, in northeastern Venezuela. The gonads of *A. zebra* began to increase in size when individuals attained 18–20 mm in shell length, but maximum gonad mass relative to somatic mass was only attained at 55–60 mm in length. Large individuals (>50 mm) showed a distinct annual reproductive cycle with a marked increase in gonad mass from July to late September 2002, coinciding with the increase in temperatures related to stratification of the water column. A decrease in gonad size occurred during October 2002 through January 2003 and coincided with a temperature decline related to renewed upwelling. The mass of somatic tissues was highest between late August to late December 2002, coinciding with the reproductive period and elevated temperatures. Other environmental factors showed little seasonal variation, although chlorophyll *a* concentration was lowest during the major period of increase in the gonad and somatic tissues (July to late September 2002), suggesting that the animals were not limited by the abundance of phytoplankton food. *A. zebra* seemed to be well adapted to the high loads of inorganic seston found in the Chacopata region.

**KEY WORDS:** reproduction, bivalve *Arca zebra*, Venezuela, temperature

### INTRODUCTION

Both environmental factors and endogenous processes control reproductive activities in marine bivalves (Bernard 1983, Griffiths and Griffiths 1987, Thompson and MacDonald 1991). Temperature, which affects rates of many physiological processes, is the environmental factor that is most often indicated to influence reproduction in marine invertebrates (Kinne 1970). In many species, particular gametogenetic phases are associated with specific temperature conditions, and temperature changes have been indicated to stimulate maturation and spawning (Giese & Pearse 1974, Roman et al., 2001). Another important environmental factor is food availability. Energetic costs for reproduction (which are usually high) depend on food ingested, or on reserves built up during periods of high food availability (Bayne & Newell 1983, Barber & Blake 1991). Many studies have examined how reproductive activities are related to food availability, stored reserves and various environmental factors (particularly temperature and salinity) in temperate bivalve species (Mackie 1984). However, we have a much poorer understanding of such relationships in tropical species because far fewer studies have been made of tropical species (Lodeiros & Himmelman 2000).

In this study we examine changes in the mass of the gonads and somatic tissues of the tropical zebra ark shell *Arca zebra* (Swainson, 1833) throughout a year and relate the changes to environmental factors. *A. zebra*, which commonly forms dense beds on rocky bottoms between 1 and 20 m in depth, is distributed along western shores of the Atlantic Ocean from North Carolina and Bermuda to Brazil (Lodeiros et al. 1999). A large natural bed of ark shells occurs in the region of Chacopata, on the Araya Peninsula, in northeastern Venezuela. This bed covers an area of 70–80 Km<sup>2</sup> and has been intensively harvested by local fishermen for

over 25 y. About 40,000 tons are harvested per year and the income provided is second to that of the sardine in Venezuelan artisanal fisheries (Mendoza 1999, Lodeiros et al. 2005). *A. zebra* is a protandric hermaphrodite, with individuals measuring 30–65 mm (anterior-posterior axis) being predominately male, 65–80 mm individuals being male or female in equal numbers and >80 mm individuals being predominately female (Nakal 1979, García 1987). The gonads are undeveloped in 20–30 mm individuals. Gametogenesis appears to be continuous, but there are peaks in July, October and March (Mora 1985, Álvarez 1992, Saint-Aubyn et al. 1992). Relationships between the reproductive cycle and environmental changes have not been previously examined. Knowledge of such relationships is needed to develop methods for producing ark shells in a hatchery. Further, an understanding of seasonal cycles in somatic and gonad tissues is required to develop a rational strategy for the management of the ark shell fishery.

### MATERIAL AND METHODS

At monthly intervals from June 2002 to July 2003, we collected samples of ark shells *A. zebra* from 3–4 m in depth from the natural bed at Chacopata (longitude between 63°46' and 63°54' and latitude between 10°42' and 10°46'). Each sample was transported (for a period of 40–60 min) in insulated boxes (at 5°C to 8°C to prevent spawning) to the Centro de Investigaciones Ecológicas de Guayacán (Universidad de Oriente) where the animals were dissected.

In the laboratory, we divided the bivalves into four groups, based on shell length (anterior-posterior axis): 10–30 mm, 30–50 mm, 50–70 mm and 70–90 mm. We analyzed 20 individuals for each size group. For each ark shell, we first removed the byssus and secondly removed epibionts and detritus from the shells. We then dissected the bivalve into three components, shell, gonad and somatic tissues. The gonad and somatic tissues were dried at 60°C

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for 72 h prior to recording their dry mass. The mass of the gonad as a percentage of the mass of the somatic tissues was calculated as a gonad index.

At weekly intervals, we also took three water samples just above the beds (at 3–4 m depth) with a 5-l Niskin bottle. From each sample we first took subsamples for determinations of the oxygen concentration (Winkler method) and salinity (induction method). Then the sample was passed through a 280- $\mu$ m filter to remove macroplankton. Finally, samples were taken for determinations of the chlorophyll *a* concentration (using the spectrophotometric method, Strickland & Parsons 1972) and of the mass of organic and inorganic materials in the seston (gravimetric method). A Sealog thermograph (Vemco Ltd., Halifax, Canada) was placed on the bottom and set to record the temperature at 10-min intervals. As the thermograph only functioned from July to December 2002, we also obtained estimates of surface temperatures from satellite images that were available from the Institute of Marine Remote Sensing of the University of South Florida, USA ([http://imars.usf.edu/sst/index\\_rm.html](http://imars.usf.edu/sst/index_rm.html)). We calculated mean temperatures for intervals of three days (including night and day images, but excluding cloudy period). These images were obtained with advanced very high resolution radiometer (AVHRR) sensors on a NOAA satellite.

From these images, sea surface temperature was extracted using the closest pixel to our study site. A time series of sea surface temperature was created, which was low pass filtered using of moving average with a window of three. When a cloud covered the pixel corresponding to our study site, we used the average of two pixels upstream and two downstream from our site (the mean current goes from east to west) and when this approach wasn't suitable, a gap was left. Finally, we fitted all data using the MATLAB cubic splineinterpolation function (a commercial package produced by Mathsoft: [www.mathsoft.com](http://www.mathsoft.com)).

For the mass of the gonad and somatic tissues of each of the four size groups we first applied a 1-way ANOVA to test for variations in size over time, and then followed with a *a posteriori* Duncan tests to identify the periods when significant ( $P < 0.05$ ) changes occurred.

## RESULTS

Each of the four size groups of ark shells *A. zebra* showed changes in somatic tissue mass and gonad mass over time (one factor ANOVAs,  $P < 0.05$ ). The seasonal changes in somatic tissue mass were greatest for the two larger size groups that measured 50–70 mm and 70–90 mm in shell length, respectively (Fig. 1). The mass increased from late June through September 2002, decreased sharply in October 2002 (ANOVA, Duncan test,  $P < 0.05$ ), increased in over the next 2–3 mo and then decreased again during January to February 2003. After this decrease values remained low until the last month of our study (late May to late June 2003) when another increase occurred. The somatic tissue mass of 30–50 mm individuals often showed a different pattern. For example, there was a significant decrease during the first month of the study and no decrease in the last month (Duncan test,  $P < 0.05$ ,  $P > 0.05$ , respectively). However, a decrease did occur during January 2003 (Duncan test,  $P < 0.05$ ), which coincided with the decrease in the largest size groups. Finally, the smallest group, individuals measuring 10–30 mm, showed much less variation in tissue mass than the larger individuals.

The gonad mass of the two largest groups of ark shells, mea-

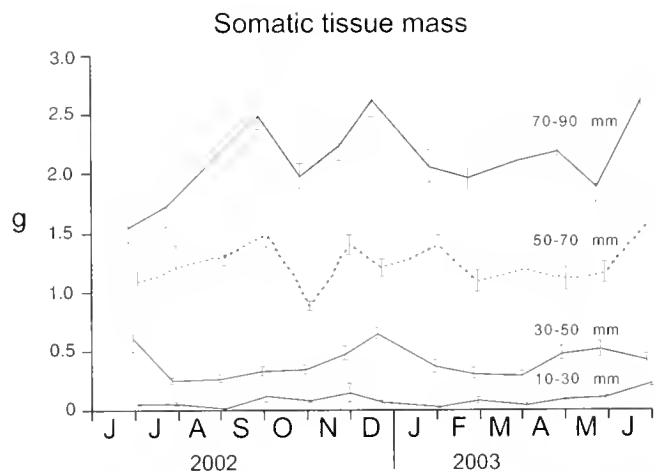


Figure 1. Seasonal changes in the mass of somatic tissues for 4 size groups (12–30 mm, 30–50 mm, 50–70 mm and 70–90 mm in shell length) of the zebra ark shell, *Arca zebra*, at Chacopata, in northeastern Venezuela. Vertical lines indicate standard errors.

suring 50–70 mm and 70–90 mm in shell length, respectively, increased rapidly during the first months of the study to a peak in late September 2002, and then a major decrease occurred during October 2002 through January 2003 (Fig. 2). After this decrease, the gonads remained small until the last month (June 2003) when renewed growth occurred. Surprisingly, the gonad mass was simi-

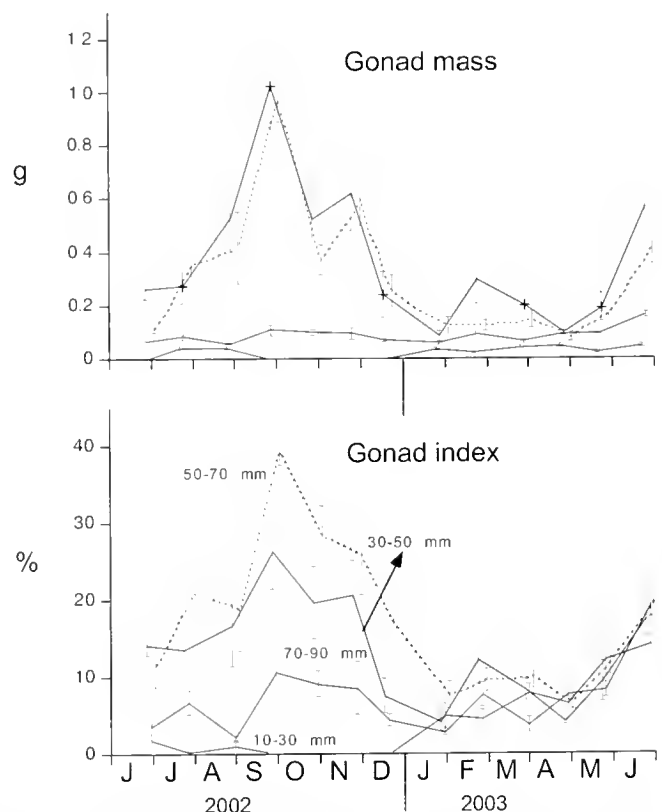


Figure 2. Seasonal changes in gonad mass and in the gonad index (gonad mass as a percentage of somatic tissue mass) for 4 size groups (12–30 mm, 30–50 mm, 50–70 mm and 70–90 mm in shell length) of the zebra ark shell, *Arca zebra*, at Chacopata, in northeastern Venezuela. Vertical lines indicate standard errors.

lar for 50–70 mm and 70–90 mm individuals (ANOVA,  $P > 0.05$ ) on most sampling dates. As a result, relative gonad mass to somatic tissue mass (the gonad index) was usually greater for 50–70 mm individuals than for 70–90 mm individuals (Fig. 2, ANOVA, Duncan test,  $P < 0.05$ ), especially in the period of major reproductive activity. Gonad mass of the two smallest groups of ark shells, measuring 10–30 mm and 30–50 mm, respectively, was always small (Fig. 2). Nevertheless, the relative gonad mass of 30–50-mm individuals showed seasonal variations, and the values during the last three months of our study were similar to those of larger (>50 mm) individuals. The gonad index of 10–30 mm individuals was negligible until February 2003, but then the values increased and followed a pattern similar to that in larger animals (with a marked increase during the last two months, May and June 2003).

The relation of gonad mass and the gonad index (gonad mass as a percentage of somatic tissue mass) to shell length for all individuals collected during our study provided information on the size at sexual maturity (Fig. 3). Although gonad mass was consistently low in ark shells up to a shell length of about 50 mm, the gonad index increased at 18–20 mm in shell length (up to 32%), indicating the onset of gonad production. Nevertheless, full maturity was not attained until a much larger size. For example, numerous individuals up to 37-mm in shell length had indices near 0%. Individuals measuring greater than 50–55 mm in length appeared to be fully mature, because what they had was wide spread in indices with few individuals with low indices.

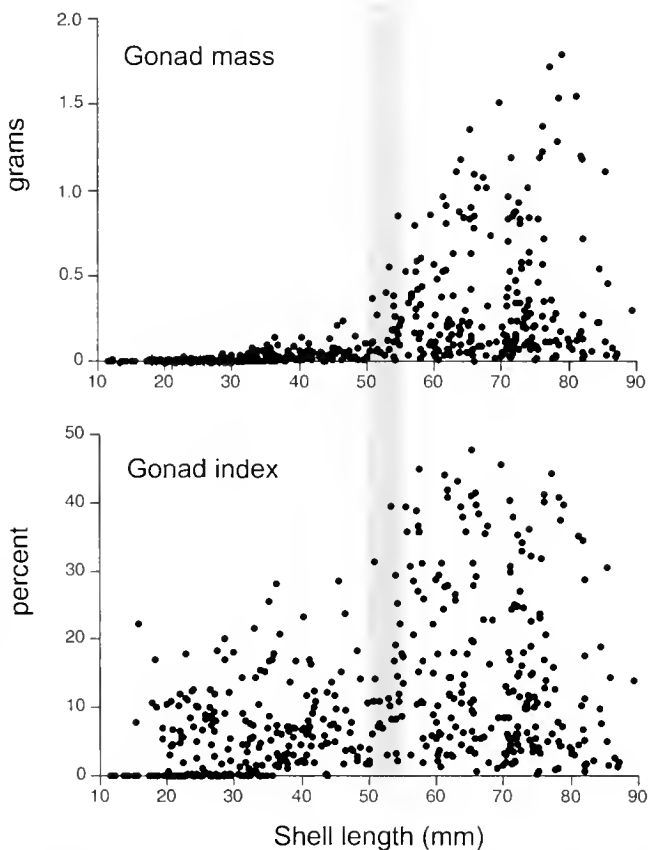


Figure 3. Relation of gonad mass and of the gonad index to shell length for the zebra ark shell, *Arca zebra*, at Chacopata, in northeastern Venezuela.

Although the thermograph we placed on the ark shell bed only functioned from July to December 2002, the record was sufficient to show that the temperature record from the NOAA satellite provided a good approximation of temperatures at our study site (Fig. 4). (The satellite data were also similar to values recorded by a thermograph at Turpialito, in the Golfo de Cariaco, 10°26'56" N; 64°02'00" W; Urbano et al. 2005). Temperatures showed a progressive increase from about 24°C in July 2002 to a peak near 27°C in October and November 2002 and this corresponded to the period of stratification of the water column. Then temperatures declined to about 23°C, indicating renewed coastal upwelling, and the lowest temperature (21.3°C) was recorded in mid March 2003. After the lowest temperature was recorded, temperatures showed an upward trend until June 2003, although with considerable fluctuations indicating irregular upwelling events.

We did not observe seasonal changes in salinity. Values were generally near the yearly mean (36.7‰), although increases by up to 1‰ were recorded on a few dates (Fig. 4). Oxygen concentrations were generally high, 3 and 5 mg/L, and the only indication of a seasonal pattern was the slight decrease in oxygen levels between August and December 2002 (Fig. 4).

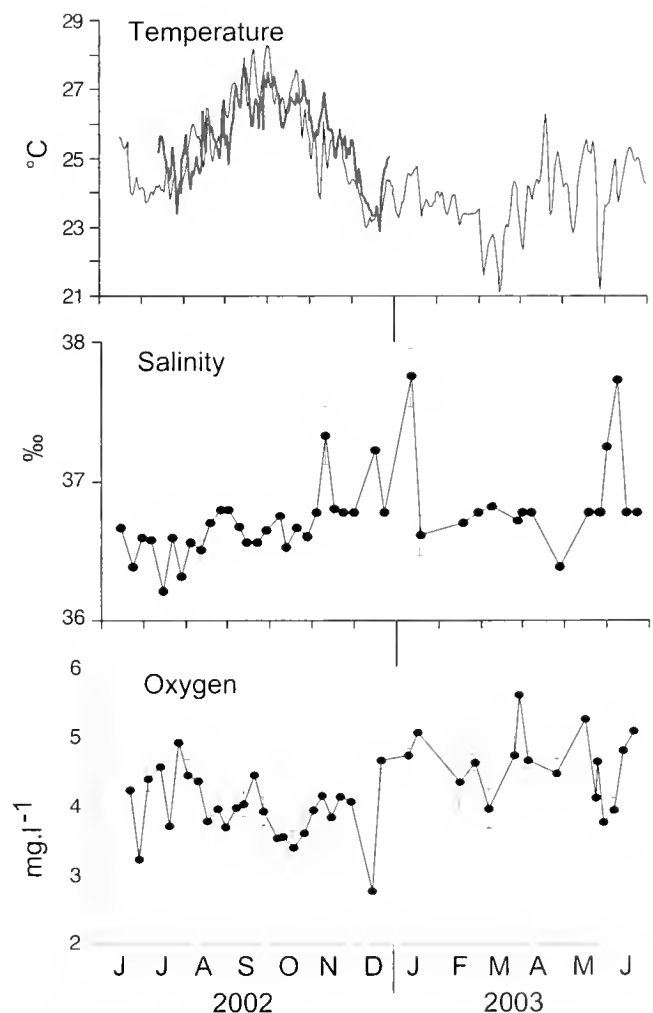


Figure 4. Variations in temperature, as recorded by a thermograph (grey line) and as estimated from satellite images and measurements of salinity and oxygen during the period from June 2002 to June 2003 at Chacopata, in northeastern Venezuela.

The mass of total seston varied from 20–40 mg/L over the study period and showed no distinct seasonal pattern (Fig. 5). An increased variation in values during the last four months of the study coincided with the increased temperature variations during the same period. The mass of organic seston also showed no seasonal pattern, and values only ranged from 5–8 mg/L. The proportion of inorganic seston to total seston was high, generally >70%. The concentration of chlorophyll *a* (Fig. 5) was usually in the vicinity of 1  $\mu\text{g/L}$ , although sporadic increases to 2.5–5.0  $\mu\text{g/L}$  were recorded. The consistently low values recorded between late July and early November 2002, coincided with the period of high temperatures associated with the stratification of the water column.

### DISCUSSION

The relation of the gonad index to shell length for the *A. zebra* showed that although the gonads begin to develop at 18–20 mm in shell length, full sexual maturity was only attained at 50–55 mm. The observations of seasonal changes in gonad indices for the four different size groups of ark shells further showed that 10–30 mm individuals had negligible reproductive output, and that 30–50 mm individuals had substantially less output than larger individuals. Individuals measuring 50–70 mm had high reproductive output, because gonad mass was similar to that of 70–90 mm individuals, and relative gonad mass was greater than that of 70–90 mm individuals.

The marked increase in gonad mass of fully mature arc shells (>50 mm) between July and late September 2002 suggested a major period of reproductive activity. This agrees with the reports of Nakal (1980) and Álvarez (1992) that the gonads of both male and female arc shells are in advanced gametogenesis stages during these months. In contrast, Saint-Aubyn et al. (1992) observed that

maximum gonad mass of *A. zebra* during 1991 was in July. Such variations likely reflect interannual differences in environmental factors, particularly factors associated with coastal upwelling, which is driven by the trade winds (Okuda et al. 1978, Lodeiros & Himmelman 2000).

The annual gonad cycle of *A. zebra* at Chacopata coincided with the seasonal changes in temperature. Both the major gonad increase from July to late September in 2002, and the beginning of a second increase during June 2003, coincided with periods of warming. Also, the decline in gonad mass from October 2002 through January 2003, likely indicating gamete release and decreased gonad production, occurred during declining temperatures. The period of minimal gonad size from February to May coincided with low temperatures. A similar positive association between reproductive activities and temperature is found in two other bivalves in northeastern Venezuela, *Perna perna* (Vélez & Lodeiros 1990) and *Lima scabra* (Lodeiros & Himmelman 1999). In contrast, in the same region, a negative relationship between reproductive activities and temperature is found in the scallop *Envula ziczac* (Lodeiros & Himmelman 2000) and the pen shell *Pinna carnea* (Narváez et al. 2000), and there seems to be no association between reproduction and temperature in the bivalves *Pinctada imbricata* (Jiménez et al., 2000), *Nodipecten nodosus* (Vélez et al., 1987) and *Pteria colymbus* (Marquez et al., 2000). Such variable relationships between reproductive activities and the annual temperature cycle in same region indicate that the different species have adopted different cues for coordinating gametogenesis events and spawning and likely contrasting strategies for providing energy for reproduction. We predict that a similar diversity of reproductive strategies exists within other invertebrate groups in the region.

The clearly annual reproductive cycle *A. zebra* was not correlated with other environmental factors, which generally showed little seasonal variation. For example, salinities and oxygen concentrations varied around the annual mean during most of the year. Also, the cycle in the mass of gonad and somatic tissues showed no relationship with food availability. In fact, the major increase in the gonad and somatic tissues in September 2002 coincided with the period of lowest chlorophyll *a* concentrations and below average levels of organic seston. These observations suggest that *A. zebra* in the Chacopata region is not limited by food availability. The inorganic content of the seston was always high in the Chacopata region (>70% of total dry seston mass) and a contributing factor could be the resuspension of materials caused by the extensive bivalve harvesting activities using drags (40,000 tons are harvested per year). Inorganic particles are often reported to be detrimental to the feeding of bivalves, because useful food particles can be strongly diluted by nonuseful particles (Riisgard 1988). However, the massive size of the arc shell population in the region, suggests that *A. zebra* is well adapted to the high levels of inorganic particles. This may be because of its ability to markedly increase its organic intake (by 31%) by selecting food particles, as reported by Ward and MacDonald (1996).

We show that the annual pattern of reproductive activity in *A. zebra* is positively associated with the temperature cycle, with the increased activity during August to December, which coincides with increased temperatures caused by stratification of the water column. This indicates that control of temperature will be an important in developing methods for producing spat in a hatchery. As the present rules controlling the arc shell fishery only limit har-

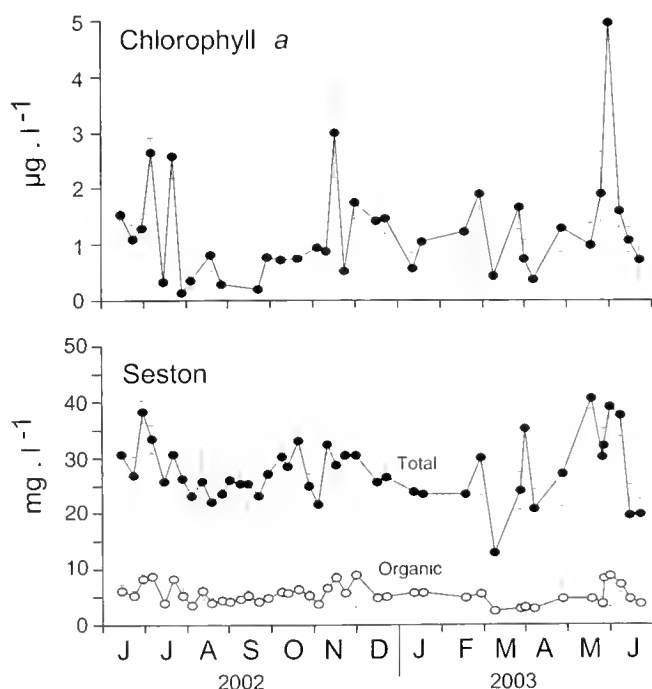


Figure 5. Variations in phytoplankton abundance as indicated by chlorophyll *a* concentration, and in the mass of total seston and organic seston during the period from June 2002 to June 2003 at Chacopata, in northeastern Venezuela.

vesting during May to August (Novoa et al. 1998), the stocks are being harvested during the reproductive period. This strategy clearly provides greater tissue mass to the fishery, but it may also limit the production of new individuals. Surprising, an extremely high biomass of arc shells has been taken from the Chacopata bed for more than 25 y. Studies are required to provide insights into the mechanisms permitting this unusually high productivity and sustainability.

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## NON-INVASIVE METHOD TO OBTAIN DNA FROM FRESHWATER MUSSELS (BIVALVIA: UNIONIDAE)

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**ABSTRACT** To determine whether DNA could be isolated from tissues obtained by brush-swabbing the mantle, viscera and foot, mantle-clips and swabbed cells were obtained from eight *Quadrula pustulosa* (Lea, 1831). DNA yields from clips and swabbings were 447.0 and 975.3 ng/ $\mu$ L, respectively. Furthermore, comparisons of sequences from the ND-1 mitochondrial gene region showed a 100% sequence agreement of DNA from cells obtained by clips and swabs. To determine the number of swabs needed to obtain adequate yields of DNA for analyses, the viscera and feet of 5 *Q. pustulosa* each were successively swabbed 2, 4 and 6 times. DNA yields from the 2, 4 and 6 swabbed mussel groups were 399.4, 833.8 and 852.6 ng/ $\mu$ L, respectively. ND-1 sequences from the lowest yield still provided 846–901 bp for the ND-1 region. Nevertheless, to ensure adequate DNA yield from cell samples obtained by swabbing, we recommend that 4 swab-strokes of the viscera and foot be obtained. The use of integumental swabbing for collection of cells for determination of genetic relationships among freshwater mussels is noninvasive, when compared with tissue collection by mantle-clipping. Therefore, its use is recommended for freshwater mussels, especially state-protected or federally listed mussel species.

**KEY WORDS:** Unionidae, genetics, integument, swabbing, mantle-clipping

### INTRODUCTION

The use of mantle-clipping for biopsy has become a common technique for collection of tissues from unionoid mussels for genetic analyses (Bubay et al. 2002, Eackles & King 2002, Jones et al. 2004, Cuore et al. 2004, Campbell et al. 2005, Geist & Kuehn 2005, Grobler et al. 2006). Berg et al. (1995) observe no significant differences in mortality rates in mantle-biopsied versus nonbiopsied mussels, *Actinonaias ligamentina* (Lamarck, 1819) and mapleleaves, *Quadrula quadrula* (Rafinesque, 1820). However, mantle-clipped snuffboxes, *Epioblasma triquetra* (Rafinesque, 1820), showed a mortality rate of 56.3% ( $n = 16$ ) after 1.5 y of postbiopsy observation at the Aquatic Wildlife Conservation Center (AWCC) (Eckert, N., VDGIF, Marion, Virginia, pers. comm.). Although mortality of mantle-clipped mussels may not be attributed directly to tissue removal, inspection of the dead *E. triquetra* showed regression of the nacre and shell deformity in the valve locations where mantle edges were removed (Fig. 1). Because mantle biopsy is an invasive procedure that may induce mortality, its use on federally endangered mussel species is a questionable procedure for genetic analyses. The goal of this study was to determine whether a procedure less invasive than mantle biopsy is available for collection of DNA for genetic analyses. Our objective was to determine whether viable DNA could be obtained by integumental swabbing from pimplebacks, *Quadrula pustulosa* (Lea, 1831) and to confirm mtDNA sequence agreement among tissues obtained from swabbing and mantle-clipping from the same mussels.

### MATERIALS AND METHODS

#### Tissue Collection

Tissues were obtained from *Q. pustulosa* at the Freshwater Mollusk Conservation Center, Virginia Tech, Blacksburg, Virginia

( $\bar{x} = 70.7$  mm,  $s = \pm 11.7$ ). To test the feasibility of isolating DNA by swabbing of the viscera, foot and mantle, we initially sampled both mantle clips and brush swabs from 8 *Q. pustulosa*. Clips (approximately 3  $\times$  5 mm) were taken from the edge of the mantle and stored in 95% ethanol. Cell samples were taken by using approximately 8 vigorous strokes with a bristle brush (CYB-1; Gentra Systems, Minneapolis, Minnesota). Strokes covered all structures within the visceral cavity, including the mantle surface, viscera and foot. Brush tips were stored in lyses buffer. Then, we determined (1) the amount of DNA that can be obtained using a bristle brush, compared with conventional mantle clips; (2) whether ND-1 could be amplified from the DNA and (3) whether ND-1 sequences amplified using buccal swabs was identical to results from mantle clips.

Because excessive scrubbing with bristle brushes resulted in some disruption of the mantle, we also investigated a collection technique using the viscera and foot only. Furthermore, it would be informative to determine the minimum number of integumental swabs required to provide an adequate amount of mtDNA for sequence analyses. For this objective, we sampled an additional 15 individuals of *Q. pustulosa*, using 6 passes of the brush on each of 5 individuals, 4 passes on another 5, and 2 passes on the 5 remaining mussels. Care was taken to rotate the brush between strokes and thus present a clean surface for collection at each stroke. After determining the amount of DNA obtained using each number of strokes, the ND-1 gene region was sequenced from the least invasive technique (2 strokes) to test the feasibility of using this as a source of DNA for sequencing reactions.

#### DNA Extraction

DNA was extracted from all samples using the Purgene DNA extraction kit. For extraction from brushes, the protocol described by Purgene was modified by increasing the initial amount of lyses buffer used from 300  $\mu$ L to 450  $\mu$ L, to ensure complete coverage of brush-bristles in a 1.5 mL Eppendorf tube. DNA was extracted

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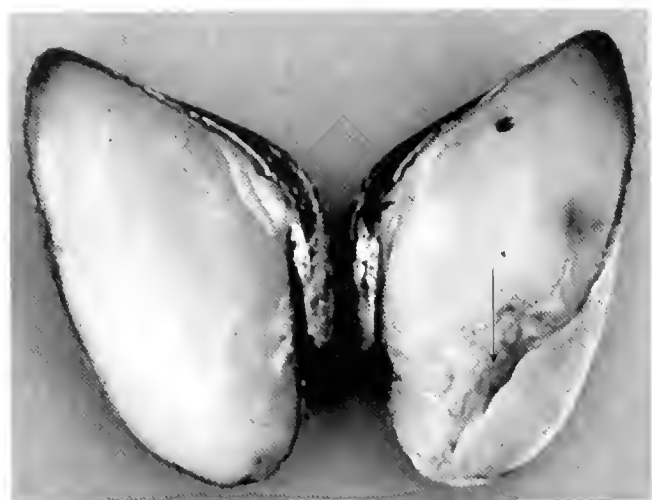


Figure 1. Shell of *Epioblasma triquetra* showing regression of nacre in the location of mantle edge removal (arrow), following postmantle clipping observation for 1.5 y.

from mantle-clipped tissue following the standard Puregene protocol for solid tissue.

#### Molecular Analysis

We used the NADH dehydrogenase (ND-1) mitochondrial gene region to verify that DNA obtained from swabs was indeed mussel DNA and that it was suitable for sequencing reactions. Primer sequences and polymerase chain reaction (PCR) amplification conditions were as reported by Buhay et al. (2002) and Serb et al. (2003). Primer sequences were forward: 5'-TGGCAGAAAAGTGCATCAGATTAAAGC-3'; reverse 5'-CCTGCTTGGAAGGCAAGTGTACT-3'. PCR reaction mixtures contained 100 ng of genomic DNA, 1× PCR buffer, 4.0 mM MgCl<sub>2</sub>, 0.4 mM dNTPs, 1.0 μM of each primer and 1.5 U AmpliTaq Gold DNA polymerase, with ddH<sub>2</sub>O added to a total volume of 25 μL. The thermal cycler profile consisted of an initial 95°C for 8 min; followed by 35 cycles of: 94°C for 40 sec, 50°C for 60 sec and 72°C for 90 sec; with a final extension step at 72°C for 2 min; and a final hold at 4°C.

PCR products were purified using a Qiagen DNA Purification kit and were sequenced using Applied Biosystems Big Dye v.3.1. Reaction mixtures consisted of 30 ng PCR product, 5-μM primer, 2 uL Big Dye 3.1 (diluted 1:1), with ddH<sub>2</sub>O added for a final volume of 7.5 uL. The cycle reaction consisted of 30 cycles, each of 94°C for 30 sec, 50°C for 15 sec and 60°C for 4 min. The products were cleaned by centrifuging through hydrated Sephadex (Sigma) in Millipore filter plates, dried down and resuspended in Hi-Di Formamide (Applied Biosystems). The samples were denatured at 95°C for 5 min, and cooled to 4°C for 2 min before being loaded on the Applied Biosystems DNA Analyzer 3730 for processing. SEQUENCHER software (ver. 4.11) was used to align and edit sequences from stored GENESCAN files.

#### RESULTS

The average DNA yields obtained varied by collection technique (Table 1). The standardized collection method from mantle clips yielded an average of 447.0 ng/μL. Eight vigorous passes of the bristle brush over the viscera and mantle yielded an average of 975.3 ng/μL (with high deviations in yield), whereas swabs of the

viscera and foot yielded 852.6, 833.8 and 399.4 ng/μL using 6, 4 and 2 strokes, respectively (and with lower standard deviations compared with strokes involving the mantle). During DNA isolation, the amount of protein in mucous-derived samples was very high, and care had to be taken to ensure full precipitation of proteins before continuing with the isolation procedure. It was necessary to repeat the protein precipitation stage of the Puregene procedure in some instances.

Sequences for the ND-1 gene region obtained from mantle clips and integument swab samples from the same three individuals aligned with 100% accuracy (847–895 basepairs; Genbank accession numbers DQ640237 to DQ640239). Sequences of ND-1 for DNA isolated after 2 strokes of the bristle brush yielded a clean 846–901 bp for the ND-1 region in two random samples (Genbank accession numbers DQ640240 and DQ640241).

#### DISCUSSION

Comparison of our data shows that swabbing of the foot and viscera of *Q. pustulosa* is a reliable method for collection of cells for DNA analyses. The DNA obtained from the swabbing technique was pure mussel DNA; our analyses show that it is of high quality, with 100% sequence alignment with DNA obtained from mantle clips from the same mussel specimens. The DNA yield from cells collected by swabbing was equal or greater than the yield from mantle clips. Our data show that 2, 4 and 6 swab passes over the foot and viscera provided sufficient quantities of DNA for reliable analyses. Because the DNA yield remains relatively constant with 4 and 6 swabbings (852.6 and 833.8 ng/μL), and then declines with 2 (399.4 ng/μL), we recommend that 4 swabs of the foot and integument be used as a standard method for collection of cells. The number of swabs required to provide sufficient cell samples for analyses may vary by mussel size and species. We recommend that researchers use this swabbing protocol rather than tissue collection by mantle-clipping, because it is noninvasive and provides reliable DNA for genetic analyses. This is especially true for federally listed mussels, typically restricted from intentional sacrifice in federal collection permits.

We observed a high protein yield in the swabbed samples that could possibly interfere with the results of future DNA analyses. We hypothesize that this protein was included in integumental mucous that was sampled inadvertently with swabbed cells. We recommend special care be exhibited during the protein-precipitation step of DNA isolations from swabbed samples.

TABLE 1.

Results of DNA extraction from mantle and integument samples.  
n = number of mussels tested.

Cell Collection Technique	Tissue Source	Number of Swab Strokes	n	Mean DNA Yield (ng/μl)
Mantle clipping	Mantle		8	447.0 ± 143.3
Integument swabbing	Viscera and mantle	8	8	975.6 ± 779.5
	Viscera and foot	6	5	852.6 ± 237.4
	Viscera and foot	4	5	833.8 ± 317.0
	Viscera and foot	2	5	399.4 ± 83.5

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## EFFECT OF TEMPERATURE, SEXUAL MATURITY AND SEX ON GROWTH, FOOD INTAKE AND GROSS GROWTH EFFICIENCY IN THE “PULPITO” *OCTOPUS TEHUELCHUS* (D’ORBIGNY, 1834)

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**ABSTRACT** The effects of temperature (10 °C and 15 °C), sexual maturity (immature and mature) and sex on instantaneous growth rates, daily feeding rates and gross growth efficiency were studied in the “Pulpito,” *Octopus tehuelchus* (d’Orbigny, 1834), under laboratory conditions. At 15 °C, immature octopus showed higher instantaneous growth and daily feeding rates than individuals maintained at 10 °C ( $P < 0.01$ ). At 10 °C, immature individuals showed higher daily feeding rates than mature specimens ( $P < 0.01$ ). Aside from a lack of measurable growth observed in mature males, sex appears to have had no effect on either instantaneous growth or daily feeding rates in either maturity stage or temperature treatment ( $P > 0.05$ ). An alternative method to compare gross growth efficiencies (GGE) among 6 treatments, based on the relationship between growth and food intake, is proposed. Immature octopuses at 10 °C showed a higher GGE value than at 15 °C ( $P < 0.05$ ). Mature individuals at 10 °C were less efficient in using food in growth than immature ones. GGE value (52%) found for immature octopus at 10 °C is among the highest reported in the literature. This could have important implications for a possible rearing activity of this species. Mature males and females at 10 °C showed a lower GGE (35.5%) than immature individuals held at 10 °C (GGE = 52%) and 15 °C (GGE = 44%). Food intake, growth and food conversion are linked processes, which in *O. tehuelchus* are influenced by temperature and sexual maturity. In addition, whereas classic statistical tests failed to detect any of these effects on GGE, the alternative method here proposed based on the relationship of growth versus food intake was successful.

**KEY WORDS:** *Octopus tehuelchus*, gross growth efficiency, food intake, temperature, sexual maturity, Southwestern Atlantic

### INTRODUCTION

The “Pulpito,” *Octopus tehuelchus* (d’Orbigny, 1834) has an important role in octopus artisanal fishery in Argentina. The main capture period takes place from December to March–April with an official annual mean capture of 20 tons (Ré 1998 a). *O. tehuelchus* is distributed from Porto Seguro (17 °S), Brazil to San Jorge Gulf (45°S), Argentina and has an intertidal distribution from San Blas Bay (40°S) to Rawson (43 °S), Argentina (Ré, 1998b). This species has a marked seasonal growth, increasing its food intake activity and growth rates as temperature increases and down these rates when temperature decreases with means of  $10 \pm 1^\circ\text{C}$  for the colder months and  $15 \pm 1^\circ\text{C}$  for the warmer months. *O. tehuelchus* has a life-span of 18 mo of duration with dorsal mantle length and body weight of first maturation for males and females of 38mm-18g and 52mm-40g respectively (Ré, 1989). Despite its economical importance to rural communities, there is no information about the effects of temperature, sexual maturity and sex on growth, food intake and gross growth efficiency under laboratory conditions in terms of a possible complementary rearing activity. Such basic studies are necessary to understand the relationship between environment conditions on food intake, growth and life-span of a species in both natural and laboratory conditions.

In cephalopods, growth and food intake are strongly dependent on a suite of biotic and abiotic variables. Water temperature and ration levels are implicated as factors most obviously responsible for variations in growth (Mangold 1983, Forsythe & Toll 1991). Final size of male and female cephalopods may vary greatly among individuals of the same species, depending on factors such as food availability and quality, and temperature (Van Heukelem 1979). Such differences in the final size typically become dramatic only in mid to later life with the approach of sexual maturity

(Forsythe & Van Heukelem 1987). In addition, there also appears to be much individual variation in growth rate and final size even within groups or siblings reared under identical conditions (Semens et al. 2004).

The cephalopod literature contains few studies reporting relationships between growth and food intake. In octopus, the functional relationship between growth and food intake appear to be linear above maintenance requirement (Van Heukelem 1976, Joll 1977), and it depends mainly on the organism’s efficiency at converting food into tissue growth and on physiological constraints. This relationship has been studied in laboratory conditions for only three tropical-subtropical species, *Octopus maya* (Voss/Solis Ramírez 1966), *O. cyanea* (Gray, 1849) (Van Heukelem 1976) and *O. tetricus* (Gould, 1852) (Joll 1977); no information is available for temperate species.

For many purposes, one of the most useful energetic measures is the gross growth efficiency (GGE), which expresses the amount of food intake required to fuel a unit amount of growth. It should be recognized, however, that any value calculated for GGE will vary with circumstances (temperature, locomotor activity, and similar) and is also dependent on food intake itself (specific dynamic action, maintenance ration) (Wells & Clarke 1996). The simplest way to resolve this discrepancy is to compare the growth of animals at different ration levels and estimate the maintenance ration in terms of the weight of food is required to maintain a stable weight (O’Dor & Wells 1987). With this information we would be able to calculate the partial growth efficiency (PGE), which is the efficiency of the organism at converting food into growth, after the maintenance requirements have been satisfied (Kleiber 1961). In young stages of cephalopods very high relative growth rates may be achieved and although feeding rates increase with temperature, there is no empirical evidence that GGE change detectably with temperature (Mangold & Boletzky 1973, Van Heukelem 1987). Aguado Giménez and García García (2002) by mean

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of a multiple regression analysis observed in *O. vulgaris* (Cuvier 1797) a temperature dependence of GGE. Considering that GGE is a ratio of two variables (growth/food intake): are the classics statistics tests adequate to detect such differences between two or more GGE samples measured on two or more conditions? Moreover, could the great individual variability reported in cephalopods reduce the probability to detect differences between GGE under different conditions?

The aims of this work are to study the effect of temperature, sexual maturity and sex on the growth, food intake and gross growth efficiency of the octopus, *Octopus tchuelchus*, under laboratory conditions. Specifically, we wanted to investigate the effectiveness of the relationship between growth and food intake as an alternative method to identify the response of gross growth efficiency to temperature, sexual maturity and sex. Finally, to compare the results of this alternative method with those obtained using the classics statistics test.

## MATERIALS AND METHODS

### Experimental Conditions

Octopuses were collected from tidepools in the intertidal zone of Puerto Lobos (42°S, 46°06'W, San Matías gulf, Argentina). Immature specimens were collected in November (late spring) 2001 and mature specimens were collected in March (early autumn) 2001. In the laboratory, octopuses were acclimatized in glass aquaria for 14 days. Octopuses were classified as immature when their body weights were lower than 18 g for males and lower than 34 g for females. For each group of immature and mature individuals, 4 experimental conditions were established: (1) males and (2) females at a 10°C water temperature, (3) males and (4) females at a 15°C temperature. Every 10 days, food intake and growth were measured over a period of 50 days for mature octopuses and 30 days for immature ones. Note the high mortality and escapement resulted in two entire missing treatments (i.e., mature males and females held at 15°C). Therefore, 6 treatments were used instead of the 8 originally proposed. For each of the 6 treatments, 5 isolated octopuses per aquaria were used. Before the end of the immature period an unbalanced design was the result of 2 immature males dead kept at 15°C. Because of this set back, only 11 records of food intake and growth were available to compare with the other 5 treatments. In consequence, to perform the statistic tests that require a balanced design, 11 measurements pairs food intake-growth were randomly chosen from each of the others 5 treatments.

Over the whole trial the light regimen was of 8 h light and 16 h darkness, and octopuses were fed daily on a monospecific diet with live crab (*Cyrtograpsus altimanus*), which has a good acceptability in laboratory conditions (Ré & Gómez Simes 1992). Each octopus was fed with one crab, which was chosen randomly with a minimum of 3% and a maximum of 17% of octopus's body weight. The effective percentage offered to each octopus was defined as a daily ration for that day. Any predated crabs were replaced with crabs of known wet weight. The weights of live crabs remaining and the unconsumed remains of predated crabs were recorded to establish the weight of tissue ingested.

### Food Intake

The amount of food intake by each octopus at intervals of 10 days was calculated for immature and mature individuals over

periods of 30 and 50 days respectively. The individual daily feeding rates were calculated following Choe (1966):

$$DFR = FI/(t * W) * 100$$

where *FI* is the total food intake (in grams) over time period (*t* = 10 days); *W* is the average of the weight measured at the beginning and end of each 10-day period. ANOVA was used to compare DFR between treatments. Because the two missing treatments (i.e., mature males and females held at 15°C) two analysis of variance tests were performed to evaluate the effects of temperature and sex on DFR in immature individuals and the effects of sexual maturity and sex on DFR in individuals kept at 10°C. In all cases the number of observations was 11 per treatment.

### Growth Measurements

To monitor individual change in wet weight, all specimens were weighed at intervals of 10 days throughout the trial. Specimens were removed from their aquaria and weighed in a plastic beaker on a digital balance (Mettler, model PC 440) to the nearest centigram. The octopuses were not anaesthetized, and the period of handling during weighing was kept brief to minimize stress to the animal. Least-squares linear regression was used to calculate instantaneous growth rates (*G*), expressed as the percent increase in body mass per day (Forsythe & Van Heukelem 1987). *G* values were compared among treatments by a parallelism test (Zar 1996).

### Growth Versus Food Intake

Growth and food intake (as a percentage of body weight) were calculated at 10 days intervals to study the relationship between growth and food intake under the experimental conditions. For each octopus, these values were calculated as follows:

$$FI(\%) = (FI/BW_i) * 100,$$

where *BW<sub>i</sub>* is the octopus wet weight (in grams) at the beginning of each time interval, and:

$$\Delta BW(\%) = (\Delta WB/BW_i) * 100,$$

where  $\Delta BW$  is the difference between the weight at the beginning and end of each time period (*t* = 10 days).

Following Van Heukelem (1976) and Joll (1977), the relationship between  $\Delta BW(\%)$  versus *FI*(%) was investigated using the linear model:

$$\Delta BW(\%) = b * FI(\%) + a \quad (1)$$

where *b* is the slope and *a* is the intercept, which estimates the weight lost at starvation condition (i.e., when food intake is equal to zero). Note that *a<sub>i</sub>* will always be negative because this is an estimate of the weight lost at starvation condition. The slopes and intercepts were compared among treatments using parallelism and ANCOVA tests (Zar 1996) respectively.

For each treatment, the maintenance level (*ML*) was calculated from Eq. 1 (as a percentage of body weight) as:

$$ML(\%) = -a/b \quad (2)$$

This follows the recommendation of Wells and Clarke (1996). From *ML*(%) definition, Eq. (1) can be rewritten using *ML*(%) as a new parameter in the function:

$$\Delta BW(\%) = b * [FI(\%) - ML(\%)]$$

Following the definition of Kleiber (1961), *b* is the partial growth

efficiency (PGE) of conversion of the food remaining after the maintenance requirements have been satisfied, then:

$$\text{PGE} = \Delta\text{BW}(\%) / [\text{FI}(\%) - \text{ML}(\%)]$$

#### Gross Growth Efficiency

The gross growth efficiency (GGE) was calculated following Choe (1966):

$$\text{GGE} = [\Delta\text{BW}/\text{FI}] \times 100 \quad (4)$$

where  $\Delta\text{BW}$  is the change in wet weight (in grams) and  $\text{FI}$  is the food intake (in grams) over each period of 10 days. The variance of GGE estimates were obtained using the delta method (Seber 1982), to include the variability caused by  $\Delta\text{BW}$  and  $\text{FI}$  estimation.

Using the relationship between  $\Delta\text{BW}(\%)$  and  $\text{FI}(\%)$ , an alternative criterion can be defined that could allow comparing gross growth efficiencies calculated under two or more treatments. Let  $T_1$  and  $T_2$  be two treatments under the relationship between food intake and growth is estimated, then from Eq.(1):

$$\begin{aligned} \Delta\text{BW}(\%)_1 &= b_1 \times \text{FI}(\%)_1 - a_1 \quad \text{and} \quad \Delta\text{BW}(\%)_2 \\ &= b_2 \times \text{FI}(\%)_2 - a_2 \end{aligned}$$

If  $b_1 = b_2$  then, for an equal food intake value of  $\text{FI}(\%)$  the change in wet weight  $\Delta\text{BW}(\%)$  values under treatments  $T_1$  and  $T_2$  will depend on intercepts  $a_1$  and  $a_2$  because:

If

$$-a_1 > -a_2 \quad \text{then,} \quad \Delta\text{BW}(\%)_1 > \Delta\text{BW}(\%)_2,$$

therefore:

$$\begin{aligned} \text{GGE}_1 &= [\Delta\text{BW}(\%)_1 / \text{FI}(\%)] \times 100 > \text{GGE}_2 \\ &= [\Delta\text{BW}(\%)_2 / \text{FI}(\%)] \times 100 \end{aligned}$$

Thus, a difference between  $a_1$  and  $a_2$  will indicate a difference between gross growth efficiencies calculated under treatments  $T_1$  and  $T_2$ . The gross growth efficiencies were compared among treatments by means of the criterion established earlier. The results obtained with this alternative method, were compared with those obtained using the classical statistical approach: (a) a direct comparison between intervals of confidence at 95% of GGE for immature octopuses kept at 10°C and 15°C, and (b) a t'Welch approximation test for disparate variances to compare GGE of immature and mature octopuses kept at 10°C.

Because a marked difference exists between the process of gain and loss weight, only positive values were used to investigate the effects of temperature, sex and sexual maturity on GGE. In fact, the effect of temperature, sex and sexual maturity on weight loss will be studied using the ANCOVA analysis.

## RESULTS

### Food Intake

Means of daily feeding rates (DFR) obtained for males and females, temperature levels and sexual maturity stages are shown in Table 1. Immature octopus of both sexes showed significantly higher DFR when held at a higher temperature value (15°C) than those held at a lower temperature value (10°C) ( $P = 0.0001$ ), whereas no effect of sex was found ( $P = 0.1301$ ) on DFR. Mature individuals at 10°C had a significantly lower daily feeding rate than immature octopuses held at the same temperature ( $P = 0.0312$ ). There was no sex-based difference between DFR of ma-

TABLE 1.

Daily feeding rate mean values obtained for mature *Octopus tchuelchus* held at 10°C and immature specimens held at 10°C and 15°C.

Temperature (°C)	Immature			Mature		
	Males	Females	Both sexes	Males	Females	Both sexes
10	1.42	1.46	1.44	0.93	1.25	1.09
15	1.96	2.47	2.21	—	—	—
10 and 15	1.69	1.96	1.82 <sup>±</sup>			

For all treatments  $N = 11$ .

(<sup>±</sup>) = Overall mean; (—) = No value is presented because of both scaping and high mortality in these treatments.

ture and immature animals of the same maturation stages when kept at 10°C ( $P = 0.2457$ ).

### Growth

By means of adjusted R square criterion, for both immature individuals and mature females, the best fit to the observed growth over time was an exponential curve, whereas mature males showed a linear growth as a best fit (Fig. 1). The slopes of the regressions for all treatments were positive. When each slope, as the instantaneous growth rate (G), was tested for significance of difference from zero, the slopes for immature individuals under 10°C and 15°C, and mature females held at 10°C, were all significantly different from zero ( $P < 0.05$ ), whereas the slope for mature males was not ( $P = 0.7780$ ). Immature octopuses held at 15°C grew faster than those at 10°C ( $P = 0.0061$ ). No differences were found between G values of immature individuals at 10°C and mature females kept at the same temperature ( $P = 0.1561$ ). The values of instantaneous growth rates for immature individuals at 10°C and 15°C and mature octopuses at 10°C are showed in Table 2.

### Growth Versus Food Intake

For all treatments the best fit for the relationship between growth and food intake was linear (Fig. 2 and Fig. 3) and Table 3. The slope  $b$ , as the partial growth efficiency (PGE) for each experimental condition was positive and significantly different from zero (Table 3). Parallelism tests indicated no difference between PGE of immature octopuses at 10°C and 15°C ( $P = 0.5972$ ). For mature individuals, the parallelism test showed no difference between PGE of males and females kept at 10°C ( $P = 0.7618$ ).

These results indicate that when maintenance requirements were satisfied, an equal portion from food remaining was invested in growth by immature octopus at 10°C and 15°C. The same situation was observed between mature males and females kept at 10°C. For immature individuals, weight lost (WL) as the percentage of body weight for an interval of 10 days was significantly higher in octopus kept at 15°C than those at 10°C ( $P = 0.0352$ ). No difference was found between WL for males and females at mature stage kept at 10°C ( $P = 0.3075$ ). The maintenance level (ML) in immature octopuses at 15°C was greater than those at 10°C and no effect of sex was observed on ML. There was no difference between ML of males and females at mature stage kept at 10°C. Results of simple regression analyses are showed in Table 3.

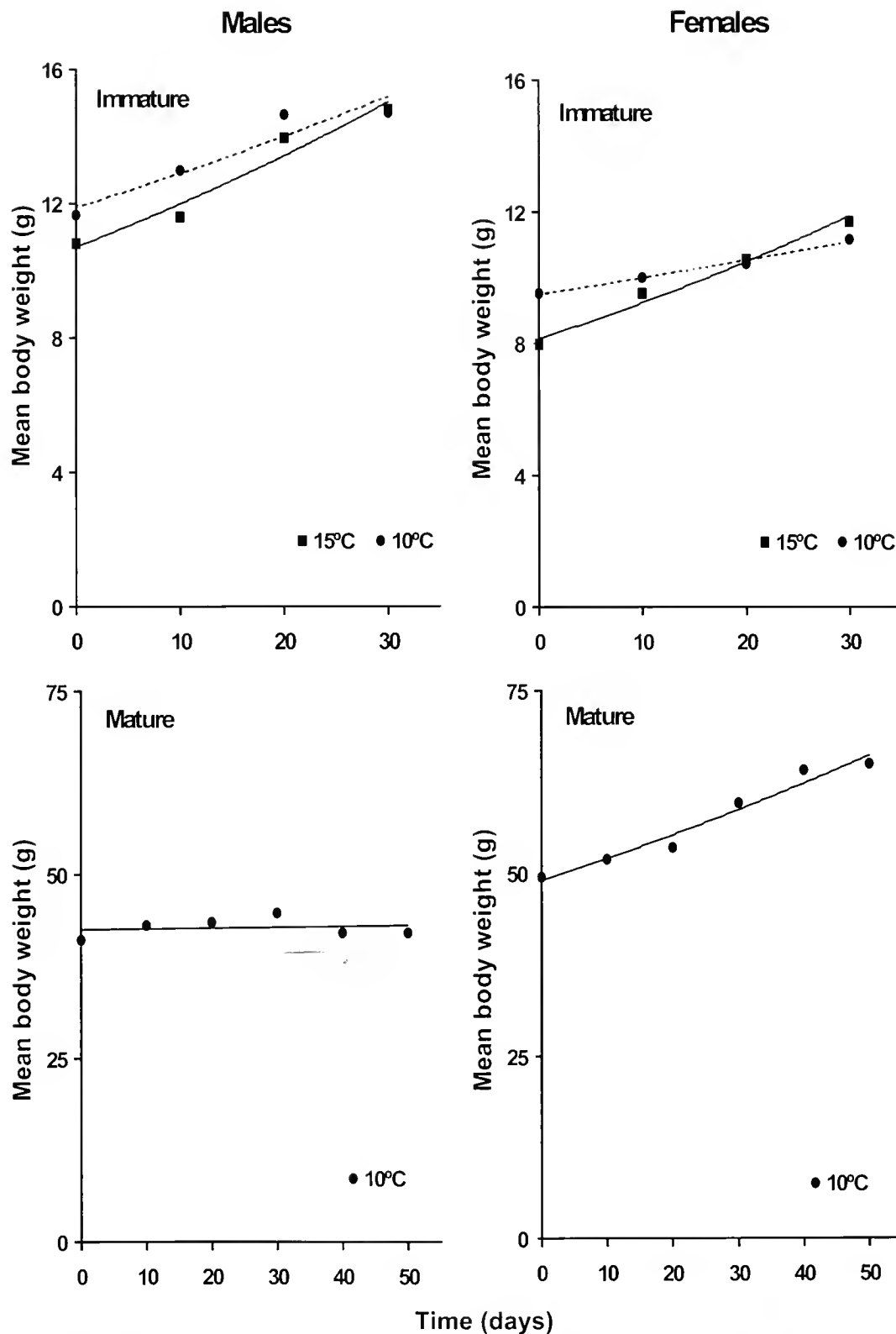


Figure 1. Mean body weight ( $n = 5$ ) of *O. tehuacensis* in grams for each treatment versus time in days. Both dash and solid lines represent the estimated curve for each treatment.

#### Gross Growth Efficiency

Gross growth efficiency (GGE) mean values indicated that immature octopuses of both sexes at 15°C invested 44% from total

ingested food in growth, whereas 52% of ingested food was invested in growth at 10°C. When negative growth data were excluded from the analysis, GGE mean value for mature individuals of both sexes kept at 10°C was 35.5% of total food intake. A



TABLE 2.

Instantaneous growth rates (G) estimated for mature *O. tehuelchus* held at 10°C and immature specimens held at 10°C and 15°C.

Temperature (°C)	Immature			Mature		
	Males	Females	Both sexes	Males	Females	Both sexes
10	0.78	0.53	0.67	0.05	0.60	0.32
15	1.05	1.28	1.19	—	—	—
10 and 15	0.97	0.89	0.93*	—	—	—

(\*) = Overall mean; (—) = No value is presented because of both scaping and high mortality in these treatments.

significant individual variation in GGE was observed for all analyzed treatments (Table 4). No difference between the variance of GGE for immature individuals at 10°C and 15°C was found ( $P > 0.05$ ). However, GGE variance of mature octopuses kept at 10°C was significantly different from that of immature individuals calculated under the same temperature treatment ( $P < 0.001$ ).

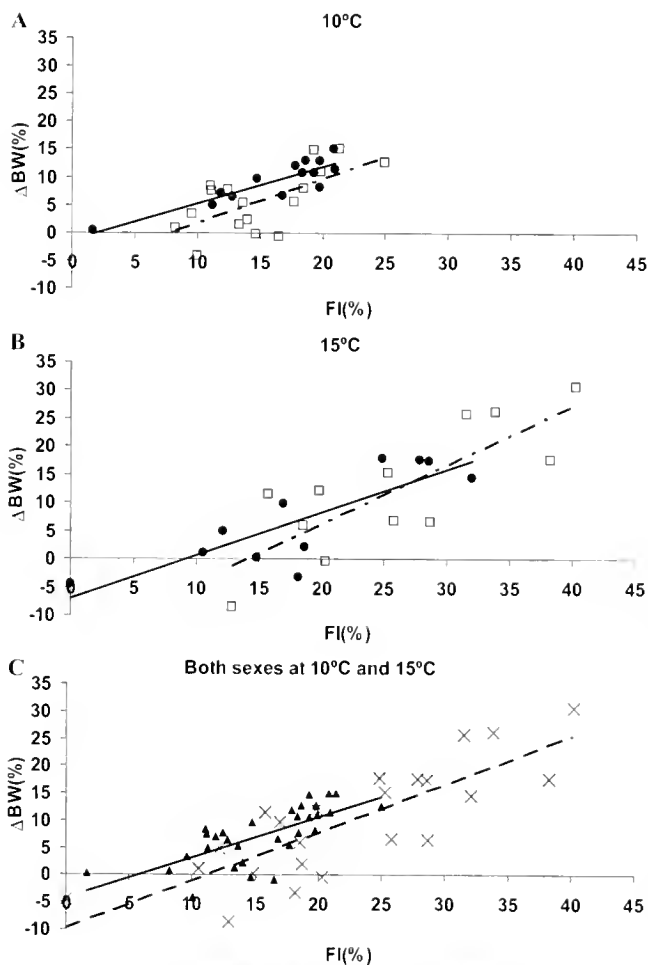


Figure 2. Growth [ $\Delta BW(\%)$ ] versus food intake [ $FI(\%)$ ] as a percentages of body weight measured each 10 days over 30 days period in males (●, solid line) and females (□, dash line) immature of *O. tehuelchus* held at 10°C (A) and 15°C (B). Pooled data for immature octopuses by temperature levels at 15°C (×, dash line) and 10°C (▲, solid line) (C). Dash and solid lines represents the estimated curve for each treatment.

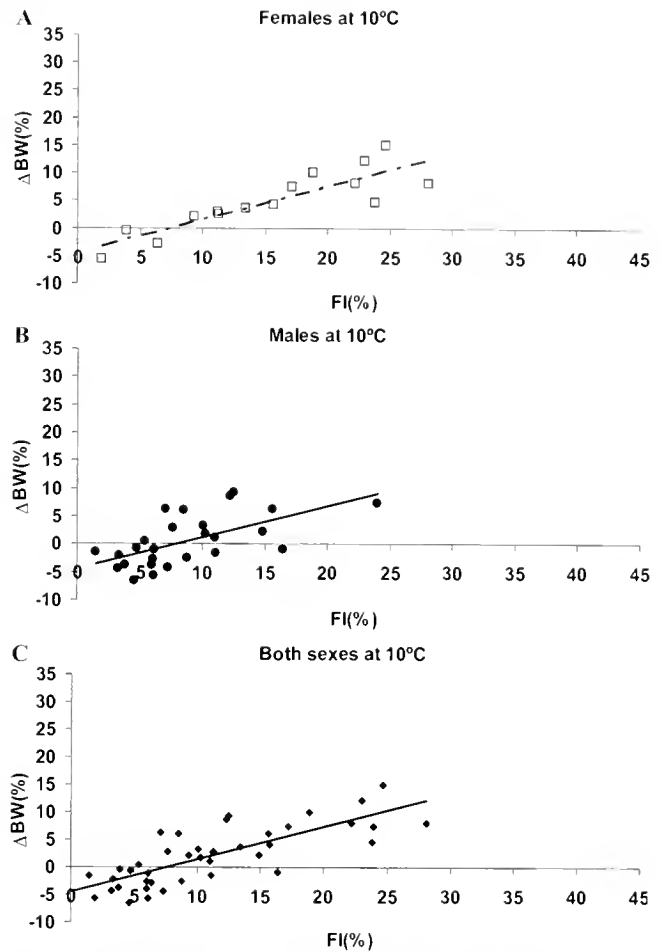


Figure 3. Growth [ $\Delta BW(\%)$ ] versus food intake [ $FI(\%)$ ] as a percentages of body weight measured each 10 days over 50 days period in both males (●) and females (□) mature of *O. tehuelchus* held at 10°C (A and B). Pooled data of males and females held at 10°C (C). Dash and solid lines represents the estimated curve for each treatment.

#### Comparison of GGE by Classical Statistical Methods

For immature octopus kept at 10°C and 15°C, direct comparison between confidence intervals at 95% indicated no significant difference in GGE mean values at different temperatures (Fig. 4). The t' Welch approximation test for disparate variances showed no differences in GGE mean values between immature and mature individuals kept both at 10°C ( $P = 0.0939$ ).

#### Comparison of GGE Based on Growth Versus Food Intake Relationship

These are the results obtained using the relationship between growth and food intake as an alternative method to investigate differences in GGE under different treatment. From intercepts analysis (ANCOVA) for immature octopuses kept at 10°C and 15°C, difference between intercepts of two temperature levels was found ( $P = 0.0326$ ), whereas no significant effect of sex was observed ( $P = 0.3695$ ). These results allow inferring in the first place that immature individuals at 10°C showed a higher GGE than those at 15°C. Secondly, under a fixed temperature males and females invested in growth the same portion of ingested food. For mature octopuses kept at 10°C, either males or females invest in

TABLE 3.

Results of regression analyses for the relationship between growth and food intake for each treatment (\*) and pooled data by sex and temperature (\*\*) in *O. tehuatlensis*.

Treatments	b (PGE)	a (WL)	a/b (ML)	n	R <sup>2</sup> <sub>adj</sub>	ANOVA (significance level)
Immature						
M at 10°C (*)	0.65	1.28	1.95	14	0.788	$P < 0.0001$
F at 10°C (*)	0.79	6.13	7.74	17	0.436	$P < 0.05$
M and F at 10°C (**)	0.75	4.28	5.7	31	0.513	$P < 0.0001$
M at 15°C (*)	0.76	7.11	9.23	11	0.687	$P < 0.01$
F at 15°C (*)	1.05	14.70	14.01	12	0.650	$P < 0.01$
M and F at 15°C (**)	0.88	9.79	11.13	23	0.668	$P < 0.01$
Mature						
M at 10°C (*)	0.56	4.35	7.80	26	0.377	$P < 0.001$
F at 10°C (*)	0.60	4.43	7.42	14	0.755	$P < 0.0001$
M and F at 10°C (**)	0.59	4.55	7.67	40	0.615	$P < 0.0001$

(M) = males; (F) = females; (PGE) = partial growth efficiency; (WL) = weight lost and (ML) = maintenance level (as the percentages of body weight) per 10 days.

growth-equivalent portions of food intake. From the intercept analysis a significant difference was detected between GGE of mature and immature octopuses kept at 10°C ( $P = 0.0020$ ). This result indicates that at 10°C immature specimens were more effective in converting food in body growth than mature ones.

### DISCUSSION

This study reported the effects of temperature, sexual maturity and sex on growth and food intake under laboratory conditions in the "Pulpito" *Octopus tehuatlensis*. In addition, an alternative method to investigate the influence of different treatments on gross growth efficiency was developed.

Within the normal range temperature adaptation of a cephalopod species, higher temperatures lead to greater food intake, with exceptions such as sepiolids (v Boletzky 1975). In addition, if food is not limiting, temperature is a key factor influencing cephalopod growth rates, particularly during the early growth phase (Forsythe

TABLE 4.

Descriptive statistics for gross growth efficiency from immature males and females of *O. tehuatlensis* held at 10°C and 15°C and mature octopus held at 10°C.

Treatments	GGE	Var (DM)	CI at 95%	Min.	Max.	n
Immature						
M and F at 10°C	52	51.20	32.78–71.74	11.02	81.73	31
M and F at 15°C	44	50.53	22.20–65.90	9.52	76.88	23
Mature						
M and F at 10°C	35.5	13.53	29.12–41.12	9.09	88.49	22

(-): negative growth data were excluded from calculation.

(M) = males; (F) = females; (GGE) = gross growth efficiency; (Var (DM)) = Estimated variance by mean of Delta Method; (CI) = confidence interval; (Min.) = minimum value; (Max.) = maximum value.

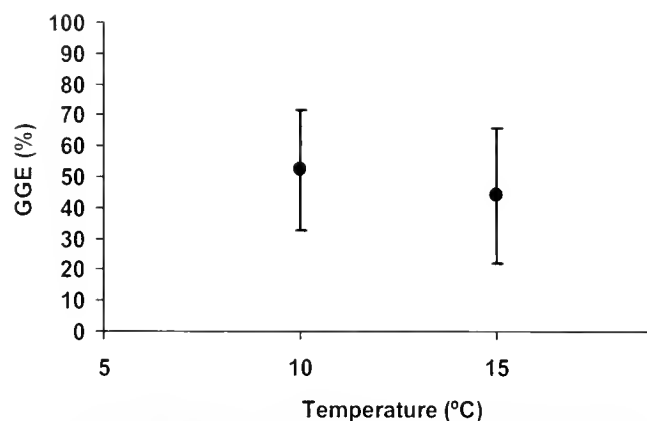


Figure 4. Gross growth efficiency (GGE) for immature *O. tehuatlensis* held at 10°C and 15°C. Vertical bars show the confidence interval at 95%.

1993). In this study, immature specimens of *O. tehuatlensis* displayed higher daily feeding and instantaneous growth rates at 15°C than 10°C. These results are in accordance with previous experimental cephalopod studies (e.g., *O. briareus*; Robson 1929, Borer 1971, *O. vulgaris*; Mangold & Boletzky 1973 and Aguado & García 2002, *Eledone moschata*; Lamarck 1798, Mangold & Boucher-Rodoni 1973, *O. tetricus*; Joll 1977, *O. ocellatus* Gray 1849, Segawa & Nomoto 2002).

A gradual daily feeding rate reduction appears to be the general rule for an octopus nearing sexual maturity (Borer 1971, Van Heukelem 1976, Wodinsky 1978). This is in agreement with the present findings for *O. tehuatlensis*, where mature males and females showed lower daily feeding rates than immature specimens kept at the same temperature.

In immature specimens of *O. tehuatlensis* the sex appears to have had no effect on feeding and growth rates; in neither 10°C nor 15°C temperature water. Mature males kept at 10°C showed a G value equal to zero whereas mature females showed a significant positive G value. This finding implies that in mature octopuses sex would have an effect on growth. Ré (1989) found that in natural conditions, *O. tehuatlensis* males reach sexual maturity three months earlier than females. Therefore, by the time those specimens were collected, males were already into an advanced sexual maturity state than females, who still were in their sexual maturation process. Moreover, although mature males and females showed no differences between daily feeding rates, only females had measurable positive growth. Likely, the lack of measurable growth in mature males of *O. tehuatlensis* could be because of an effect of advanced age on growth process, which would allow a normal feeding activity with either low or none growth-tissue production. Similar results have been reported by Cortez et al. (1995) for *O. mimus* (Gould, 1852) collected from the Pacific waters of Chile.

For the present work, no statistically significant, sex-based differences were found among immature animals in terms of instantaneous growth rates or daily feeding rates. However, a slight tendency of instantaneous growth rates was observed to show slightly higher values in males at 10°C but higher in females at 15°C. Similarly, Forsythe and Hanlon (1988) observed higher growth in *O. bimaculoides* (Pickford/McConnaughey, 1949) males at 18°C but higher in females at 23°C. Considering that males of *O. tehuatlensis* reach the sexual maturity approximately 3 mo earlier than females, this tendency in data could indicate an interaction

between sex and temperature on instantaneous growth rates and daily feeding rates for immature specimens. Thus, in colder periods, immature males should show significantly higher daily feeding rates, instantaneous growth rates and gross growth efficiencies than females.

For all treatments performed in the present study, the relationship between growth and food intake was linear. Estimated values of body weight lost and maintenance level in immature octopuses at 15 °C were higher than those at 10 °C. In mature individuals kept at 10 °C these values showed no differences between sexes. Immature individuals showed constant partial growth efficiency over sexes and temperature treatments. Because of the difference, experimental and biological conditions (e.g., temperature level and body weight) and comparisons among species become an arduous task. For individuals of *O. tetricus* kept at a mean of 20 °C, a graphic estimation from the linear relationship between growth and food intake a maintenance level of 1% BW day<sup>-1</sup> and a daily body weight lost of 0.7% were obtained. The value of partial growth efficiency was estimated as 82.8% after the maintenance requirements have been satisfied (Joll 1977). For *O. cyanea* and *O. maya* reared at 25 °C, the best fit to explain the relationship between growth and food intake was linear (Van Heukelem 1976, 1987). For both species the least square method indicated a maintenance level of 1.8% BW day<sup>-1</sup> and a body weight lost of 1.01% BW per day with a partial growth efficiency of 70% (Van Heukelem 1976, 1987). From present data and literature cited, an increase in temperature yielded higher maintenance level and daily body weight lost, whereas the linear form of the relationship between growth and food intake remained unalterable.

Gross growth efficiency (GGE) estimated for immature *O. tehuelchus* held at 10 °C (GGE = 52%) are among the highest reported in the literature. Nixon (1966) observed a GGE value of 46% for *O. vulgaris*. Research on *O. maya* and *O. cyanea* held at 25 °C produced GGE means values of 39.5% and 38.3% respectively, although for the estimated values of GGE mature octopuses data were not included (Van Heukelem 1976). Joll (1977) found that *O. tetricus* kept between 16.5 °C and 24.2 °C possess a GGE mean value of 46.8%. A lower GGE value of 37% was found for *Eledone moschata* held at 15 °C (Mangold 1987). Daly and Peck (2000), recorded for three individuals of the Antarctic octopus *Pareledone charcoti* (Joubin, 1905) at 0 °C under laboratory conditions a GGE mean value of 32%.

Gross growth efficiency declines when old males and maturing females of *O. maya* and *O. cyanea* decrease food intake (Van Heukelem 1976). Moreover, O'Dor and Wells (1978) pointed out that sexual maturation in females involves different needs for nutrients and energy, because females have higher reproductive costs

for egg formation. When the t'Welch approximation test for disparate variances was used to compare GGE means between mature and immature specimens kept at 10 °C, no significant difference was detected. Contrariwise, the alternative method based on the comparison between intercepts was able to detect a significant difference between GGE of immature and mature octopuses. Agreeing to Wells et al. (1983) and O'Dor et al. (1983) findings for *O. vulgaris*, *O. tehuelchus* showed in growth and food intake a high individual variability. Likely, this variation in GGE, which produces great variance value, can explain why t'Welch approximation test failed to detect differences between GGE. Furthermore, the low daily feeding rate and high maintenance level observed in mature specimens of *O. tehuelchus* could yield a lower GGE than immature octopuses kept at 10 °C.

The confidence intervals at 95% comparison indicated no difference between GGE of immature specimens kept at 10 °C and 15 °C. Contrariwise, the alternative method based on the comparison between intercepts indicated that immature specimens held at a 10 °C showed higher GGE than individuals kept at 15 °C. Mangold and v Boletzky (1973) for individuals of *O. vulgaris* feed *ad libitum* and held at 10 °C, 15 °C and 20 °C recorded gross growth efficiencies mean values of 56%, 55% and 48% respectively. Despite this, no statistics differences in GGE of *O. vulgaris* between temperature levels were reported. As it was observed in *O. tehuelchus*, *O. vulgaris* showed the highest GGE value at the lowest temperature treatment. Aguado Giménez and García García (2002) by mean of a multiple regression analysis observed in *O. vulgaris* that GGE increased from 13 °C to an optimal temperature value at 16.5 °C, after this point GGE decreased to zero at 23 °C. As it was observed in *O. tehuelchus* and *O. vulgaris*, for the normal range of temperature for a species, gross growth efficiency is affected by temperature. In addition, in *O. tehuelchus* the sexual maturity has an effect on GGE. The results obtained in this work indicate that, whereas classic statistical tests failed to detect the effects of temperature and sexual maturity on GGE, the alternative method based on the growth versus food intake relationship was able to do it. The information obtained about the effect of temperature and sexual maturity on growth, food intake and gross growth efficiency of *O. tehuelchus* under laboratory conditions could be used to develop potential rearing techniques.

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## SUBCELLULAR DISTRIBUTION OF Ag, Cd, Co, Cu, Fe, Mn, Pb, AND Zn IN THE DIGESTIVE GLAND OF THE COMMON CUTTLEFISH *SEPIA OFFICINALIS*

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**ABSTRACT** The subcellular fractionation of the digestive gland cells of the common cuttlefish *Sepia officinalis* was performed to investigate the distribution of metals between organelles and cytosol and the different cytosolic fractions separated by gel-filtration chromatography. Total metal concentrations vary over 3 orders of magnitude, ranging from dwt for Pb to dwt for Zn. With the exception of Cd, Co, and Cu, metals were mostly bound to the organelles. Whereas no specific organelle compartment was found for Mn, Pb, and Zn, Fe was mainly associated with nucleus, brown body, and "boule" fraction (i.e., 52%) and 44% of the total Ag was contained in the lysosomal and mitochondria enriched fraction. The link of metals with hydrosoluble proteins in the cytosolic fraction was investigated at 254 and 280 nm. Direct relationship between cytosolic metal and metallothioneins could only be established for Ag and Cu, whereas Cd and Zn seem to mainly bind high (>70 kDa) and low (<4 kDa) molecular weight proteins.

**KEY WORDS:** cephalopod, metal, trace element, detoxification, metallothionein, chromatography, *Sepia officinalis*

### INTRODUCTION

Exclusively marine organisms, cephalopods, are active predators found from polar to tropical ecosystems and from the shallow waters to very deep ocean environments. As well, they constitute a major food source for many top predators species (see the reviews by Clarke 1996, Croxall & Prince 1996, Smale 1996, Klages 1996). They therefore have a key role in many marine ecosystems and are also of increasing interest for worldwide fisheries (Amaratunga 1983, Rodhouse 1989). Despite such ecological and economical importance, metal: radioactive; and organic contaminants have globally been poorly studied in cephalopods.

Concerning metals, cephalopods concentrate several trace elements such as Ag, Cd, Cu, or Zn at sometimes very high concentrations (e.g., Martin & Flegal 1975, Miramand & Bentley 1992, Bustamante et al. 1998). Most of the studies reported the major role of the digestive gland in the bioaccumulation mechanisms, this organ suspected to have a key function in the metabolism of many metals in cephalopods (e.g., Miramand & Bentley 1992, Bustamante et al. 2002a, 2004). Thus, the digestive gland of cephalopods constitutes the main storage organ for essential (e.g., Co, Cu, Fe, and Zn) and non essential elements (e.g., Ag, Cd, Pb, and V) independently of the considered species and of its area of origin (e.g., Miramand & Guary 1980, Smith et al. 1984, Finger & Smith 1987, Miramand & Bentley 1992, Bustamante et al. 1998, 2002a, 2004, Miramand et al. 2006). Thus, around 70% to 98% of the whole body burden of metals could be readily stored in the digestive gland (Miramand & Bentley 1992, Bustamante et al. 2002b). This could result from the very long half-life of some elements like Cd and Co (Bustamante et al. 2002a, 2004) and/or to very efficient translocation processes from other organs and tissues to the digestive gland (e.g., Ag or Cs; Bustamante et al. 2004, 2006). In the case of Cd and Co for example, the residence time of the metal in the whole organism could be longer than the life span of the cephalopods, meaning that almost all the assimilated metal is definitively sequestered in the digestive gland (Bustamante et al.

2002a, 2004). In contrast to Cd and Co, Ag displays a faster turnover in cephalopods (Bustamante et al. 2004). Even the dissolved pathways are probably the main route for Ag accumulation in cephalopods, the digestive gland contains most of the whole body burdens of this metal (Miramand & Bentley 1992, Bustamante et al. 2000, 2004). This fact suggests that very efficient translocation processes allow the transfer of Ag from tissues and organs in contact with seawater to the digestive gland for detoxification purposes (Bustamante et al. 2004).

In both cases (i.e., metals directly stored for a long time in the digestive gland and metals having a peculiar tropism to this organ) the elevated concentrations reported on metal bioaccumulation in cephalopods suppose the occurrence of efficient storage and detoxification mechanisms to counteract the toxicity of metals (e.g., Simkiss & Taylor 1982, Phillips & Rainbow 1989). Detoxification mechanisms of marine invertebrates mainly involve the precipitation or co precipitation of metals on amorphous granules and the binding on proteins, which can be nonspecific (e.g., transferrin, ferritin) or specific to one or more metals (e.g., vanabins, metallothioneins [Durand et al. 2002, Ueki et al. 2003]). One well-known detoxification strategy involving proteins is the binding of some trace metals to metallothioneins (MTs), which play a role in the homeostasis of the essential metals Cu and Zn, but are induced by various other metals (i.e., Ag, Cd, and Hg) (Engel & Brouwer 1989, Cosson et al. 1991, George & Olsson 1994). Thus, MTs are considered to be involved in Ag, Cd, and Hg detoxification (Dallinger 1993, 1995, Roesijadi 1992, 1996, Viarengo & Nott 1993). In cephalopods, association of MT with metals in the digestive gland seems to mainly concern Cu and, to a lesser extent, Cd and Zn (Tanaka et al. 1983, Finger & Smith 1987). In the digestive gland, most of Cd and Zn appear to be bound to other cytosolic proteins than to MTs (Tanaka et al. 1983, Finger & Smith 1987, Castillo et al. 1990).

Considering the elevated metal concentrations occurring naturally in the digestive gland of cephalopods, the aim of our study was to investigate the metal distribution between the different organelles and the cytosol and to provide insight on the implication of hydrophilic proteins such as MTs in the detoxification mecha-

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nisms. The common cuttlefish *Sepia officinalis* was selected as an experimental model, and the subcellular distribution of various metals, Ag, Cd, Co, Cu, Fe, Mn, Pb, and Zn, were considered in mature male individuals. Finally, the cytosolic fraction obtained was chromatographed to determine the association of the different metals to the hydrophilic proteins.

## MATERIALS AND METHODS

### Biological Material

Male mature common cuttlefish were caught in the Bay of Seine (French coast of the English Channel) and kept alive at the most two days in outflow tanks. Animals belonging to the same age class, from the same sex and sexual maturity state were selected to minimize the biological variability ( $n = 4$ , total weight  $785 \pm 84$  g). Prior to the experimentation, cuttlefish were anaesthetized in seawater containing 2% ethanol and rapidly dissected. The digestive gland was carefully removed, weighed ( $n = 4$ ,  $37 \pm 10$  g), and prepared for direct heavy metal analysis and subcellular fractionation.

### Subcellular Fractionation

Aliquots ranging from 1–2 g of each individual's digestive gland were homogenized with a mortar and pestle on ice with 4 volumes of a 20-mM TRIS-HCl, 0.25-M sucrose buffer (Tanaka et al. 1983), at pH 8.6. The homogenates were successively centrifuged at 600g for 10 min, 10,000g for 10 min and 100,000g for 60 min at 4°C in a Beckman LE-70 ultracentrifuge. This procedure giving six different fractions is summarized in Figure 1. Each pellet was collected to determine the metal concentrations in the membranes, nuclei, "boules" (which are considered as heterolysosomes and heterophagosomes involved in intracellular digestion of cephalopods [Boucaud-Camou 1976, Boucaud-Camou & Yim

1980]) and brown bodies fraction, the mitochondrial and lysosomal fraction, and the microsomal fraction. The accuracy of the sequential separation was controlled by Transmission Electronic Microscopy after fixation in 4% glutaraldehyde and postfixation with osmium tetroxide in 0.4 M cacodylate buffer at pH 7.3. The particle-free supernatant fraction (cytosol) was removed for heavy metal analysis and for gel filtration chromatography.

### Gel-filtration Chromatography

Prior to the gel filtration chromatography, total proteins were quantified in the collected cytosol following Lowry et al. (1951). Then, 2 mL of this fraction were chromatographed on a Sephadex G-75 superfine ( $16 \times 800$  mm) column (Pharmacia) equilibrated and eluted with 20 mM Tris-HCl buffer, pH 8.6 at 4°C, containing 50 mM NaCl and 3 mM  $\text{NaN}_3$ . The column was maintained at 4°C, and the samples were collected as 4 mL fractions. The UV absorbance of the eluate was measured at 254 and 280 nm with a U-1100 Hitachi spectrophotometer. In each eluted fraction, the heavy metal concentrations were also determined. The column was calibrated for molecular weight estimations with Ovalbumine (43 kDa), Chymotrypsin (25 kDa), Ribonuclease (13.7 kDa), and Glucagon (3.5 kDa) as standard markers. We also used equine renal metallothionein (13.4 kDa, Kojima et al. 1976) to identify the fractions containing MTs.

### Metal Analyses

Samples of the digestive gland were previously dried at 80°C to constant weight prior to analysis. The dried digestive gland samples, the pellets, and the particle free supernatants resulting from the subcellular separation, and the different fractions separated by gel chromatography were digested with 5 mL of 14 N ultrapure  $\text{HNO}_3$  at 100°C during 3 days. After evaporation of the acid, the residues were taken up in 5 mL 0.3 N  $\text{HNO}_3$  and analyzed for Ag, Cd, Co, Cu, Fe, Mn, Pb, and Zn by flame and graphite furnace atomic absorption spectrophotometry with a Zeeman Hitachi model 180–70.

Quality control was assessed by heavy metal analyses in blanks and reference materials. Thus, Orchard-Leaves (National Bureau of Standards) and MA-A-2 fish flesh standard (IAEA) were treated and analyzed in the same way as the samples. Our results for the standard reference materials were in good agreement with the certified values (Table 1). The detection limits were ( $\mu\text{g}\cdot\text{g}^{-1}$  dry weight): 0.004 (Cd), 0.02 (Ag), 0.1 (Co, Mn, Pb), 0.5 (Cu, V, and Zn), and 2.5 (Fe). Results are also expressed in micrograms per gram of the dry tissue weight ( $\mu\text{g}\cdot\text{g}^{-1}$  dwt).

## RESULTS

### Metal Concentrations

The concentrations of Ag, Cd, Co, Cu, Fe, Mn, Pb, and Zn are shown in Figure 2. Among the analyzed metals Zn is the most concentrated, reaching up to  $600 \mu\text{g}\cdot\text{g}^{-1}$  dwt, followed by Fe ( $424 \pm 142 \mu\text{g}\cdot\text{g}^{-1}$  dwt) and Cu ( $362 \pm 114 \mu\text{g}\cdot\text{g}^{-1}$  dwt). All other elements displayed far lower concentrations (i.e., ranging from  $2.2 \mu\text{g}\cdot\text{g}^{-1}$  dwt for Pb to  $13.6 \mu\text{g}\cdot\text{g}^{-1}$  dwt for Cd).

### Subcellular Distribution

The partitioning of metals among the (1) nuclei and brown bodies; (2) lysosomes and mitochondria; (3) microsomes; and (4) soluble cytosolic fraction is presented in Table 2. Most of the Cd, Co, and Cu were associated with hydrosoluble cytosolic com-

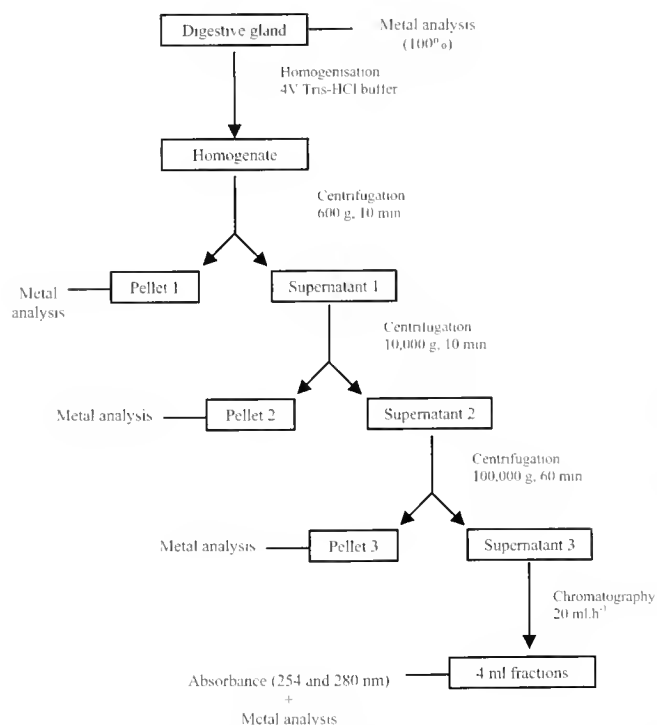


Figure 1. Procedure of the subcellular fractionation of the digestive gland of the cuttlefish *Sepia officinalis* from the Bay of Seine.

TABLE 1.

Comparison of elemental concentrations ( $\mu\text{g}\cdot\text{g}^{-1}$  dwt) of Orchard-Leaves standard, SRM 1571 (National Bureau of Standards) and fish flesh homogenate, MA-A-2 (International Agency of Atomic Energy) obtained in present study with certified values.

Standard	Ag	Cd	Co	Cu	Fe	Mn	Pb	Zn
Orchard Leaves								
Present study	—	$0.10 \pm 0.05$	$0.17 \pm 0.04$	$10 \pm 1$	$272 \pm 14$	$82 \pm 7$	$38 \pm 2$	$22 \pm 6$
Certified values	—	$0.11 \pm 0.02$	(0.2)	$12 \pm 1$	$300 \pm 20$	$91 \pm 4$	$45 \pm 3$	$25 \pm 3$
MA-A-2								
Present study	$0.12 \pm 0.01$	$0.069 \pm 0.008$	$0.09 \pm 0.04$	$3.4 \pm 0.7$	$65 \pm 5$	$0.62 \pm 0.09$	$0.43 \pm 0.14$	$35 \pm 4$
Certified values	$0.10 \pm 0.01$	$0.066 \pm 0.004$	$0.08 \pm 0.01$	$4.0 \pm 0.1$	$54 \pm 1$	$0.81 \pm 0.04$	$0.58 \pm 0.07$	$33 \pm 1$

(—): recommended value

pounds whereas Ag, Fe, Mn, Pb, and Zn were mostly bound to the organelles. Fe is mainly associated with the nuclei and brown bodies (52%), and 44% of the total Ag is contained in the lysosomal and mitochondria enriched fraction. It is noteworthy that Co, Cu, Pb, and Zn are equivalently distributed in each pellet (Table 2).

#### Metal Associated With Proteins

Chromatographic Sephadex G-75 elution profiles of the absorbance at 254 and 280 nm obtained for cytosol from the digestive gland of *Sepia officinalis* were used to determine the metal concentrations ( $\text{mg}\cdot\text{L}^{-1}$ ) in the collected fractions containing the proteins separated by their molecular weight (Fig. 3). A first peak centered on fraction 11 corresponds to the void volume (macromolecules larger than 70 kDa, such as hemocyanin), and a second peak between fractions 40 and 44 indicates a particular richness in small proteins and polypeptides (4 kDa and less). MTs used for calibration fell in fraction 26 with an elevated 254/280 nm absorbance ratio (equal to 16). All metals were associated with high and low molecular weight proteins (>70 kDa and lower than 4 kDa, respectively) except Ag, which was not detectable in the small protein fraction. Intermediate proteins ranging from 10–20 kDa did not contain detectable amounts of Fe and Mn. Ag and Cu were the only metals showing a peak in the fraction 26 region among those expected to bind MTs (Ag, Cd, Cu, and Zn). Cd and Zn displayed

a very similar distribution with a major fraction associated with high molecular weight proteins for both metals.

#### DISCUSSION

The metal concentrations measured in the digestive gland of male adult cuttlefish from our study globally fell within the range of values reported for *Sepia officinalis* from the English Channel (Miramand & Bentley 1992), the Bay of Biscay (Schippe & Hevert 1978, Bustamante 1998, Bustamante et al. 1998), and the Mediterranean (Bustamante et al. 2002a). Although globally poorly documented, metal concentrations in cephalopods have received increasing interest as these molluscs play a major role as predators and food items in marine ecosystems. The central role of the digestive gland in metal bioaccumulation has been highlighted many times, particularly for toxic metals such as Ag and Cd (e.g., Martin & Flegal 1975, Miramand & Bentley 1992, Bustamante et al. 2002a, 2004), but the detoxification processes occurring in this organ remain poorly understood (Bustamante et al. 2002b). Investigations on detoxification mechanisms have focused either on the subcellular distribution of metals between cytosol and organelles (Rocca 1969, Tanaka et al. 1983, Finger & Smith 1987, Bustamante et al. 2002b, Craig & Overnell 2003), the involvement of hydrosoluble proteins in the binding of metals (see also Decleir et al. 1978, Ueda et al. 1985, Castillo et al. 1990, Castillo & Maita 1991) or on the histochemical and microanalytical localization of metals within the digestive gland cells (Schippe & Hevert 1978, Martoja & Marcaillou 1993). Moreover, the subcellular distribution of metals mainly concerned Cd, Cu, and Zn (i.e., Rocca 1969, Decleir et al. 1978, Tanaka et al. 1983, Ueda et al. 1985, Finger & Smith 1987, Castillo et al. 1990, Castillo & Maita 1991, Bustamante et al. 2002b, Craig & Overnell 2003), but very limited information is available for other elements. To the best of our knowledge, the studies of Tanaka et al. (1983) and Finger & Smith (1987) are the only ones providing data on other metals (i.e., Ag and Fe and  $^{210}\text{Po}$ , respectively). The few studies on metal detoxification in cephalopods have considered various models like cuttlefishes (Decleir et al. 1978, Schippe & Hevert 1978, Martoja & Marcaillou 1993, Bustamante et al. 2002b), squids (Tanaka et al. 1983, Finger & Smith 1987, Castillo et al. 1990, Castillo & Maita 1991, Bustamante et al. 2002b), and octopuses (Rocca 1969, Ueda et al. 1985, Bustamante et al. 2002b). Consequently, results are often different between authors and sometimes contradictory. Therefore, there is a need to provide more information on the subcellular distribution of heavy metals in general and on poorly or not yet studied elements, also highly concentrated in the digestive gland of cephalopods. In this respect, our study provides the first

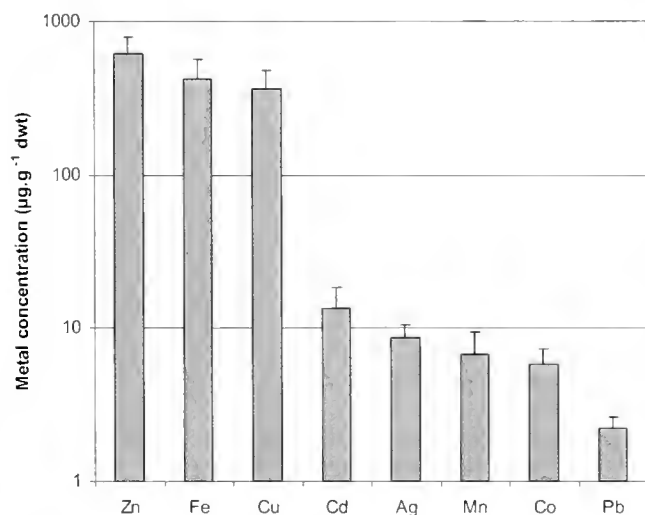


Figure 2. Levels of heavy metals ( $\mu\text{g}\cdot\text{g}^{-1}$  dwt) in the digestive gland of the cuttlefish *Sepia officinalis* from the Bay of Seine.

TABLE 2.

Partition of the metals (%) among the different separated fractions from the digestive gland homogenates of the common cuttlefish *Sepia officinalis*.

Fractions	Ag	Cd	Co	Cu	Fe	Mn	Pb	Zn
Nuclei and brown bodies	19 ± 3	14 ± 8	11 ± 6	13 ± 9	52 ± 3	10 ± 4	20 ± 9	17 ± 8
Lyosomes and mitochondria	44 ± 3	11 ± 4	13 ± 1	12 ± 2	30 ± 5	31 ± 2	20 ± 3	20 ± 2
Microsomes	13 ± 4	23 ± 5	12 ± 3	19 ± 4	14 ± 2	32 ± 9	22 ± 4	23 ± 6
Total organelles	76 ± 10	48 ± 17	36 ± 10	44 ± 15	96 ± 10	73 ± 15	62 ± 16	60 ± 16
Cytosol	24 ± 2	52 ± 9	64 ± 9	56 ± 9	4 ± 1	27 ± 5	38 ± 8	40 ± 9

insight about the subcellular distribution of Co, Mn, and Pb in cephalopods.

Our study of metal distribution between the insoluble (membranes and organelles) and soluble (cytosol) fractions of the digestive gland leads to the conclusion that metals can be separated between those mainly associated with the hydrosoluble compounds Cd, Co, and Cu and those mainly associated with the organelles Ag, Fe, Mn, Pb, and Zn. This shift does not therefore correspond to the essential or non-essential character of the metals but rather to the result of specific regulation/detoxification processes.

As already mentioned, the scarce information on the subcellular distribution of trace elements in cephalopods put forward that 50% to 90% of the Cd is usually found in the soluble fraction of the digestive gland of cephalopod from the field (Finger & Smith 1987, Castillo et al. 1990, Bustamante et al. 2002b), even if the squid *Todarodes pacificus* does not follow this trend with only 26% ± 3% of the metal being present under a soluble form (Tanaka et al. 1983). Our results for *S. officinalis* are consistent with this

general trend (Table 2), suggesting the presence of mechanisms of detoxification of Cd involving soluble proteins. The presence of Cd detoxification mechanisms involving MTs was suspected when considering the chromatograms of metalloproteins from the digestive gland of the squids *Nototodarous gouldi*, *Todarodes pacificus*, and *Onmmastrephes bartrami* from the Pacific Ocean (Tanaka et al. 1983, Finger & Smith 1987, Castillo & Manta 1991). Later, these proteins were quantified in various cephalopod species from the Northern Atlantic Ocean (Bustamante et al. 2002b). In *S. officinalis* from our study, cytosolic Cd was mainly associated with high molecular weight proteins, and MTs seem to have a minor role in the binding of this metal (Fig. 3). This result is in accordance with previous reported data for other cephalopod species. For example, most of the cytosolic Cd in the digestive gland of the squids *T. pacificus* and *Onychoteuthis borealiojaponica* was bound to proteins weighing more than 70 kDa (Tanaka et al. 1983, Castillo et al. 1990). Only a small part of the soluble Cd was bound to low molecular weight proteins (<3 kDa) or to proteins of similar size to MT (10 kDa to 16 kDa). Moreover, Finger & Smith (1987) have

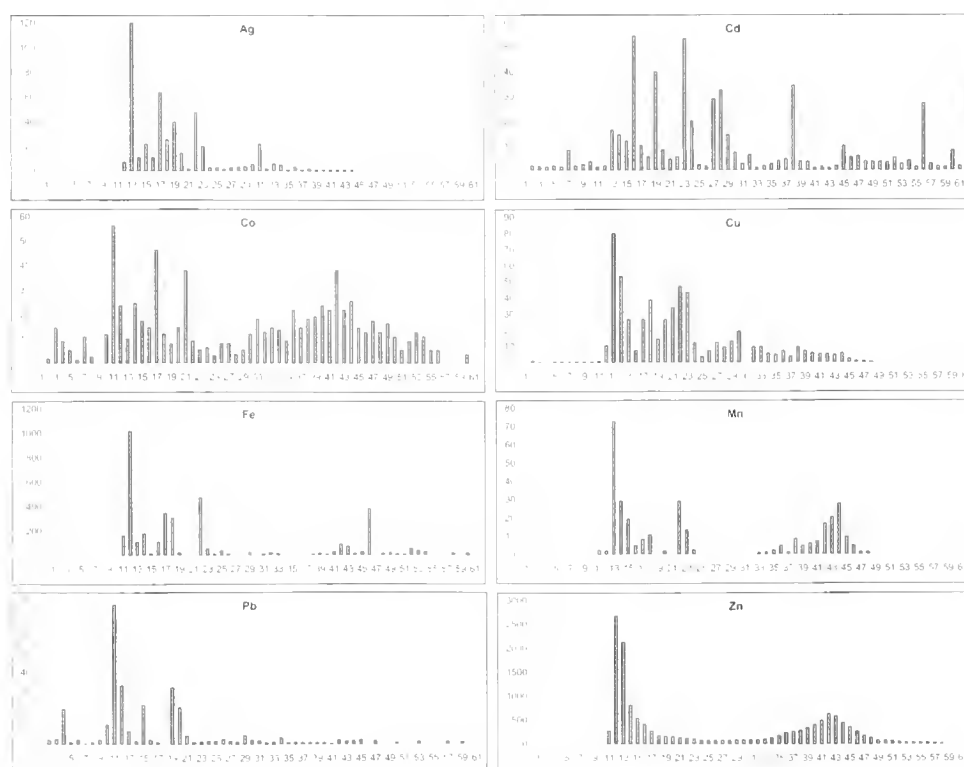


Figure 3. Metal profiles ( $\mu\text{g.L}^{-1}$ ) after Sephadex G-75 gel chromatography of the cytosolic fractions of *Sepia officinalis* digestive gland.



also reported the occurrence of Cd-binding proteins with a high molecular weight ( $\geq 70$  kDa) in the digestive gland of the squid *N. gouldi*.

Similarly to Cd, Cu has been mainly found in the cytosol of the digestive gland cells of the squids *N. gouldi* ( $78 \pm 10\%$ , Finger & Smith 1987) and *T. pacificus* ( $63 \pm 5\%$ , Tanaka et al. 1983) but poorly associated with hydrosoluble compounds in the squid *Loligo forbesi* (35%, Craig & Overnell 2003) and in the octopus *Octopus vulgaris* ( $28 \pm 17\%$ , Rocca 1969). The presence of high Cu concentrations in the digestive gland cells of *S. officinalis* has been revealed histochemically in specific structures called "spherules" (Martoja & Marcaillou 1993). These authors suggested that these metal-rich spherules would be made of complexed metallothionein-like proteins and would explain the high concentrations of Cu within the digestive gland. However, the presence of such structures has not been confirmed by other studies (Boucaud-Camou & Boucher-Rodoni 1983), and our results are not in accordance with such a hypothesis. Because of their size (i.e., several  $\mu\text{m}$ ), such structures would be expected to sedimentate in the first or the second pellet fraction, containing in fact a low proportion of the total Cu (Table 2). In *S. officinalis* Cu was mainly cytosolic, a main pool corresponding to high weight proteins that might contain hemocyanin molecules (Taylor & Anstiss 1999). However, Cu also appears to be bound to MT proteins, and to a lower extent, small size proteins and peptides (Fig. 3).

To the best of our knowledge, no data on Co subcellular distribution in the digestive gland of cephalopod have been published to date. In *S. officinalis*, 64% of this metal was associated with the cytosolic fraction, which is similar to the results reported for the Bivalves *Chlamys varia* (76%), *Gafrarium tumidum* (79%), and *Isognomon isognomon* (65%) in their digestive glands (Bustamante & Miramand 2005, Météan et al. 2005). Low molecular weight proteins seem to have a major importance in binding Co (Fig. 3). This result is consistent with those of Nakahara et al. (1982) for *O. vulgaris* exposed to  $^{60}\text{Co}$  by way of seawater, where the proteins involved in the binding of the radionuclide weighed less than 3 kDa.

The predominant distribution of Ag in the insoluble fraction (*viz.* the noncytosolic fractions) could be caused by specific Ag storage/detoxification in the digestive gland. In various bivalves, Ag is trapped as nontoxic  $\text{Ag}_2\text{S}$  precipitates within their tissues (Ballan-Dufrançais et al. 1985, Martoja et al. 1989, Berthet et al. 1990, 1992) and is mainly found associated with the organelle subcellular fraction (e.g., Bustamante & Miramand 2005). This mechanism of sequestration would therefore inhibit the potentially deleterious effects of the toxic Ag, which is highly accumulated in the digestive gland of cephalopods (Martin & Flegel 1975, Miramand & Bentley, 1992). Specifically, the lysosomal and mitochondrial fraction appears to play a major role in the binding of Ag (Table 2). Our results concerning Ag are opposite to those of Tanaka et al. (1983) for the squid *T. pacificus* for which  $64 \pm 4\%$  of the metal was reported to be soluble and associated with small proteins ( $< 3$  kDa). The reasons of such a totally contrary result are difficult to identify because various biological and environmental factors could modify the subcellular distribution of a single metal within a group or a species, such as the phylogeny, the main pathway of incorporation (seawater vs. food), the level of the metal accumulated, etc (e.g., Ueda et al. 1985, Bustamante et al. 2002b). This clearly points out the need to give light on the issue of Ag subcellular distribution using a wide range of cephalopod species and controlled experimental conditions. Within the cytosolic frac-

tion, Ag was mainly bound to high molecular weight proteins and to a lower extent to MTs.

Similarly to Ag, Pb has no biological functions. In the digestive gland of *S. officinalis*, most of this metal was found in the organelles (62%). In the digestive gland of the scallop *C. varia*, Pb was also mainly bound to organelles (i.e., 66% of the total metal burden [Bustamante & Miramand 2005]). Similarly, in *Mytilus galloprovincialis* and *Modiolus modiolus*, Pb was mainly associated with the fraction containing nuclei, cellular debris, and insoluble salts (Julshamm & Andersen 1983, Regoli & Orlando 1994). In these Bivalves, Pb is accumulated by endocytosis and precipitate as sulphur or phosphate salts inside the digestive cells (Coombs & George 1978) as well as in the extracellular compartments (Schulz-Baldes 1978). Lysosomes are deeply involved in the detoxification of Pb and lead to the formation of Pb salts representing the final stage of the lysosomal detoxification process (Simkiss 1977). In *S. officinalis* no specific affinity among the different fractions has been shown (Table 2), suggesting that such a detoxification process is relatively limited, likely to be caused by low Pb concentrations found in this species.

The subcellular distribution of Fe in *S. officinalis* with 96% of the metal being bound to organelles, can only be compared with the 86% reported for *T. pacificus* (Tanaka et al. 1983). However, the distribution among the organelle fraction is clearly different as in the cuttlefish 52% was bound to the nuclei fraction whereas in the squid, 42% was bound to the microsome fraction. In Bivalves such as mussels or clams, Fe is also primarily associated with the nuclei fraction (Julshamm & Andersen 1983, Sullivan et al. 1988, Regoli & Orlando 1994). In these molluscs, Mn has a similar subcellular distribution as Fe, which is actually not the case for cephalopods (Table 2). This difference in the distribution of Fe and Mn between the organelles could be caused by the difference in the diet between carnivorous cephalopods and suspending or deposit feeder Bivalves. Indeed, Bivalves could ingest suspended/deposited material enriched in Fe and Mn hydroxide particules (e.g., Bryan & Uysal 1978). Within the cytosolic fraction, Mn and Fe are the only elements not associated with MTs (Fig. 3).

## CONCLUSION

The subcellular distribution of heavy metals clearly varies depending on the considered element. For essential and toxic metals, the sequestration in either the organelles or the cytosolic proteins could lead to specific accumulation. In this context, it is particularly striking that the different detoxification mechanisms for toxic Ag (mainly insoluble) and Cd (largely soluble) lead to their bioaccumulation at relatively high concentrations in the digestive gland of *S. officinalis*. In our conditions, a direct relationship between cytosolic metal and MT could only be established for Ag and Cu, whereas Cd and Zn seem to mainly bind high ( $> 70$  kDa) and low ( $< 4$  kDa) molecular weight proteins. Further studies should focus on the induction of MTs by the different metals inducing it synthesis in other invertebrates (i.e., Ag, Cd, Cu, Hg, and Zn) in controlled conditions to determine the dynamic of the detoxification processes in cephalopods.

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## FECUNDITY OF *CANCER JOHNGARTHI* CARVACHO 1989 (DECAPODA: BRACHYURA: CANCRIDAE) FROM SOUTHERN BAJA CALIFORNIA'S WESTERN COAST, MEXICO

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**ABSTRACT** Fifty-two ovigerous females of *Cancer johngarthi* were caught from the Baja California Peninsula's western coast, Mexico. Carapace length, carapace width and total weight of each specimen were registered. Fecundity estimates were obtained from the wet weight of eggs in each organism. Mean fecundity was 765,227 eggs, corresponding to a mean carapace width of 117 mm, and the mean relative fecundity was 4,305 eggs per gram of female body weight. Fecundity data (F) were fitted in relation to carapace width (CW) to the power model  $F = 0.84CW^{2.87}$ ,  $R^2 = 0.52$ . *C. johngarthi* has a high population renewal capacity compared with other larger species like *Maiopsis panamensis*, which produces between 351,000 and 1,162,668 eggs, and *Stenocionops ovata*, which produces between 35,187 and 180,057 eggs; in both species, the relative fecundity is lower than 1,000 eggs per gram of female body weight.

**KEY WORDS:** *Cancer johngarthi*, fecundity, Baja California Peninsula

### INTRODUCTION

*Cancer johngarthi* is a crab belonging to the Cancridae family, distributed from Guadalupe island, Mexico (29°N, 118°W) to Panama bay (7°N) (Carvacho 1989). This species is commercially important because of the flavor and texture of chelae meat, mainly from males. In the Southern Baja California's western coast, as well as in the Gulf of California, this species lives in soft substrates, at depths in excess of 90 m, and aggregated in populations, which may sustain a fishery (Fiol-Ortiz & Cervantes-Díaz 1996, Sánchez-Ortiz et al. 1997, Carvacho 1989, Leija et al. 1992, Ramírez-Rodríguez et al. 2003). Based on these results, the Mexican government recently offered two commercial fishing permits for *C. johngarthi* using traps.

This implies the need to conduct detailed studies on the crab's biology and dynamics aimed at generating useful information for the design of fishery-management measures. For the previously mentioned, the main objective of this work is to produce fecundity estimates for *C. johngarthi*, of particular interest because of its relationship with the population renewal capacity of this species in the study area (García-Montes et al. 1987).

### MATERIALS AND METHODS

In January 2003, *C. johngarthi* specimens were caught from the Southern Baja California Peninsula's western zone (24°45' to 25°10'N; 112°31' to 112°44'W and 23°34' to 23°52' N; 111°04' to 111°04' W, Fig. 1). The capture was conducted using a 25-m long fishing ship with a net capacity of 130 t, adapted to operate conical traps measuring 150 cm × 75 cm × 65 cm, with a plastic-coated steel frame and baited with sardine at a depth between 73 and 300 m (Ramírez-Rodríguez et al. 2003).

Fecundity was determined from the wet weight of eggs in each ovigerous mass. The number of eggs in three subsamples of 0.02 g each was counted using a stereomicroscope. Individual fecundity data were obtained by extrapolating the number of eggs in each in the three subsamples to the total weight of the egg mass in each female, and then the mean per female fecundity and the standard deviation were calculated. For each specimen, fecundity data were

adjusted in relation to carapace width (CW). CW was defined by the greatest straight-line distance (excluding lateral spines) across the carapace and chelae width and carapace length (CL) was defined by the line midway between the eyes to the midpoint of the posterior margin of the carapace. Also, the relative fecundity was calculated for each female as the number of eggs per gram of female body weight.

### RESULTS

Of the three thousand eight hundred and thirty-six organisms sampled, 3,362 were males (87.6%) and 473 females (12.4%), of which 52 were ovigerous. The sex rate for the total sample was 1M:0.14F and significantly deviates from the 1:1 rate. Male size ranged from 98 mm to 176 mm in CW, with a mean of 146 mm ± 11 mm, whereas females measured 87–153 mm in CW, with a mean of 122 mm ± 9 mm.

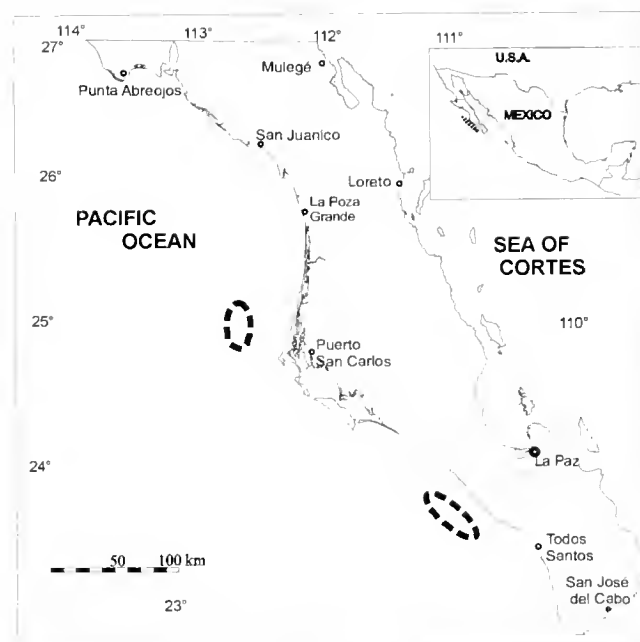


Figure 1. Baja California Peninsula's western coast. Sampling area.

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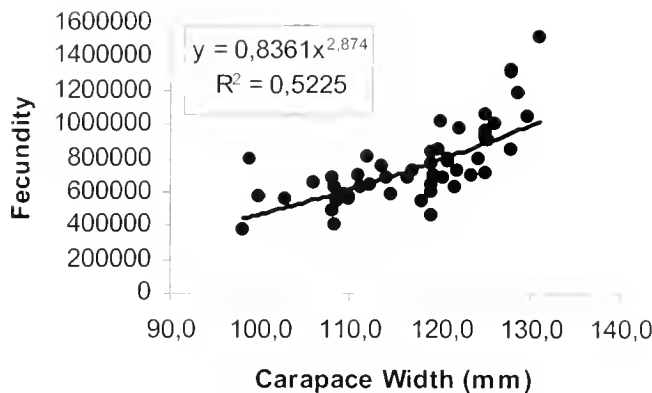


Figure 2. Potential relationship between CW and partial fecundity in *Cancer johngarthi*.

The 52 ovigerous females had a CW ranging between 98 and 131 mm, with a mean of  $117 \text{ mm} \pm 8.4 \text{ mm}$ ; CL lied between 51 and 81 mm with a mean of  $74 \text{ mm} \pm 6.9 \text{ mm}$ . Fecundity varied between 381,441 and 1,517,668 eggs, with a mean of  $765,227 \text{ eggs} \pm 233,415 \text{ eggs}$ , whereas the relative fecundity ranged between 1,033 and 8,510 eggs/g with a mean of  $4,305 \text{ eggs/g} \pm 1,402 \text{ eggs/g}$  of female body weight.

A power equation was fitted to fecundity data, with CW as the independent variable  $r^2 = 0.52$   $\alpha = 0.05$ , Fig. 2). The fit was poor in relation to CL ( $r^2 = 0.31$ ), whereas it failed to display a defined relationship with respect to chelae length and body weight. Relative fecundity also failed to show a clear relationship with CW ( $r^2 = 0.003$ , Fig. 3).

#### DISCUSSION

According to the results of this work, *C. johngarthi* is a species with a high population renewal capacity, compared with other species of larger deep crabs like *Maiopsis panamensis*, which produces between 351,501 and 1,162,668 eggs, and *Stenocionops ovata*, which produces between 35,187 and 170,057 eggs; both species have a relative fecundity lower than 1,000 eggs per gram of female body weight (Villalejo-Fuerte et al. 1998, 1999).

In crustaceans, fecundity estimates derived from incubating eggs carried by females in their abdomen yield lower numbers compared with the estimates based on the number of intragonadal oocytes, because resorption of intraovarian oocytes during the maturation process occurs frequently. Additionally, many eggs fail when adhered to the pleopods at the time of spawning (Farmer 1974). Another factor that accounts for extraovarian fecundity estimates being lower than intraovarian ones is egg predation during incubation (Abelló & Sardá 1982); however, fecundity estimates obtained from incubating-egg data yield more precise information to determine the renewal capacity of a population.

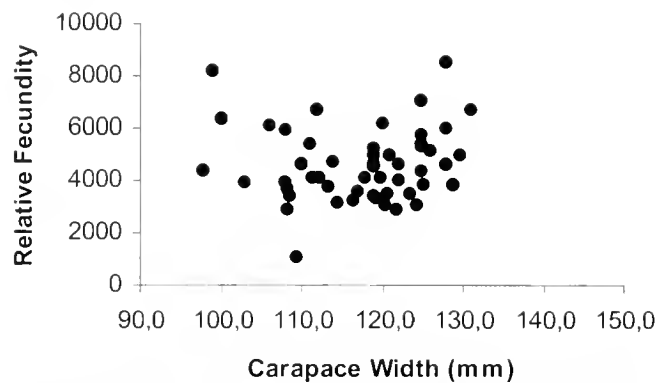


Figure 3. Relationship between CW and relative fecundity in *Cancer johngarthi*.

The ovigerous masses analyzed were evenly colored, ranging from orange to dark brown, which suggests that the spawning period takes place rapidly with embryonic development occurring synchronously, so that no difference is evident, at least to the naked eye. Histological evidence suggest that *C. johngarthi* can produce more than one batch of eggs in a breeding cycle, as is the case of the deep-living Majids *Maiopsis panamensis* and *Stenocionops ovata* (Villalejo-Fuerte et al. 1998, 1999). This would account for the low determination coefficient of the potential fit to fecundity data, because the number of eggs produced in successive partial spawning events decreases, resulting in a less adequate relationship with the organism's morphometric variables, however there is no evidence in the fecundity-length data.

According to Leija et al. (1992), the reproduction process for *C. johngarthi* takes place in October and recruitment during March and September; however, in this work ovigerous females were caught in January, so that the spawning, fertilization and incubation of eggs is likely to occur during the fall and winter, with recruitment taking place in the spring. To prove the earlier discussed, it is required to conduct sampling across a whole annual cycle, recording the frequency of ovigerous females to confirm this information.

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## POPULATION GENETIC STRUCTURE AND GENETIC DIFFERENTIATION OF *ARTEMIA PARTHENOGENETICA* IN CHINA

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**ABSTRACT** Ten strains of *Artemia parthenogenetica* have been collected from inland salt lakes and coastal salterns in China. Ten diploid clones were selected from each *Artemia parthenogenetica* strain for analysis of the population genetic structure and genetic differentiation using inter simple sequence repeats (ISSR). One hundred and seventy fragments (100–2200 bp) were generated using 20 ISSR primers. A high level of genetic variation was found, with 96.47% polymorphic loci in total loci. The number of alleles ( $N_a$ ), effective number of alleles ( $N_e$ ) and Shannon information index ( $S_i$ ), the mean Nei's gene diversity ( $H$ ), the average values of  $H_t$ ,  $H_s$ ,  $G_{st}$  for pairwise subpopulations and mean value of all 100 clones from 10 Chinese *Artemia parthenogenesis* strains were analyzed. The results showed that genetic structure of populations of *A. parthenogenetica* from China were complicated with high genetic diversity among the populations. Cluster analysis was then performed to create a dendrogram using the UPGMA method based on the Nei's genetic identity. The UPGMA dendrogram showed that 10 Chinese *Artemia parthenogenetica* strains can be significantly divided into three major groups (subpopulation): Liaoning, (PK and YK); Shandong and Hebei, (HH, TG, LN, WD, WZ and FC) and Qinghai and Xinjiang (GH and BLK).

**KEY WORDS:** *Artemia parthenogenetica*, ISSR, genetic structure, genetic differentiation

### INTRODUCTION

Brineshrimp, *Artemia* (Crustacea Anostraca), are widely distributed in inland salt lakes and coastal salterns over the world. Populations of the genus *Artemia* were found in more than 600 habitats dispersed across the world (Van Stappen 2002). The genus *Artemia* comprises a complex of bisexual species defined by the criterion of reproductive isolation and of a large number of parthenogenetic populations under the binomen *A. parthenogenetica*, composed of diploid and polyploidy individuals for taxonomic convenience (Sun et al. 1999). The Morphological study (Triantaphyllidis et al. 1994), genetic variation (Zhang & King 1992) and evolution of most parthenogenetic *Artemia* populations have been examined by means of allozyme electrophoresis, karyotype, high repeat sequence (Abreu-Grobois & Beardmore 1980, 1982, 1983, 1991; Beardmore & Abreau-Grobois 1983, Badaracco et al. 1991, Bowen et al. 1988, Barigozzi, 1974, Barigozzi et al. 1987, Hou et al. 1993, 2000), molecular markers such as RAPD and AFLP (Camargo et al. 2002, Sun et al. 2000). In China, *A. parthenogenetica* is found in either saltworks along the coast of Bohai sea or salt lake from Qinghai, Xinjiang (Wang et al. 1991; Yang et al. 1995, 1996, Hou et al. 2000). The relationship with bisexual *Artemia* and expression of isozyme gene have been analyzed (Gao et al. 1994; Hou et al. 2003), but little is currently known about that population genetic structure and genetic differentiation of different geographic parthenogenetic *Artemia* strains in China.

Molecular genetic information has been increasingly used to detect the population genetic structure and genetic diversity among morphologically similar populations of a same species. Of the many molecular approaches available today, the simple inter sequence repeats (ISSR) technique is among the most sensitive. The ISSR technique had been successfully used to reveal population

genetic structure and relationship (Kantety et al. 1995, Nagaoka & Ogihara 1997, Martín & Sánchez-Yélamo 2000) and genetic diversity (Awasthi et al. 2004; Brantestam et al. 2004). The ISSR primer sequences are designed from microsatellite regions and the annealing temperatures used are higher than those used for RAPD markers, which have better reliability. Also, the technique does not require prior knowledge of DNA sequence for primer design, which is more practical (Wolfe et al. 1998).

In this study, the molecular marker of ISSR was used to help analyze the population genetic structure and genetic diversity of *Artemia parthenogenetica* in China. The Chinese *Artemia parthenogenetic* populations along the coastal of Bohai sea are all diploid (Pilla 1992, Triantaphyllidis et al. 1997) with the exception of Huanghua (Hebei Province) where a few tetraploid individuals appeared, and there are also a few triploid, tetraploid and pentaploid individuals that appeared in Balikun saltlake and Aibi saltlake in the Xinjiang autonomous region; therefore only diploid clones are being studied.

### MATERIALS AND METHODS

*Artemia* cysts of 10 geographic strains of *A. parthenogenetica* were collected directly from different areas in China, followed by their code abbreviations (used hereafter), is as follows: Balikun, Xinjing Autonomous Region (BLK); Gahai, Qinghai Province (GH); Wudi, Shandong Province (WD); Yingkou, Liaoning Province (YK); Tanggu, Tianjin (TG); Luannan, Hebei Province (LN); Pikou, Liaoning Province (PK); Huanghua, Hebei Province (HH); Fengcheng, Shandong Province (FC); Wuzhi, Shandong Province (WZ) (Table 1).

The nauplii were hatched from the cysts of different strains according to the methods described by Sorgeloos et al. (1986). The nauplii were cultured for 15 days to adulthood in the laboratory, fed on *Dunaliella salina*, then 30 single individual clones were created from each strain according to the methods described by

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TABLE 1.  
Samples of *Artemia parthenogenetica* for present study

Abbreviation	Location	Collection Time
BLK	Balikun, Xinjing, China	2002.9
GH	Gahai, Qinghai Prov., China	1999.9
WD	Wudi, Shandong Prov., China	2002.9
YK	Yingkou, Liaoning Prov., China	2000.9
TG	Tanggu, Tianjin, China	2001.9
LN	Luannan, Hebei Prov., China	2000.9
PK	Pikou, Liaoning Prov. China	2002.9
HH	Huanghua, Hebei Prov., China	2002.9
FC	Fengcheng, Shandong Prov. China	2001.9
WZ	Wuzhi, Shandong Prov., China	2002.9

Hou et al. (2000), and 300 clones were obtained from 10 strains. The ploidy of clones was examined using the methods described by Cai and Hou (1991) and Yang et al. (1996). 10 diploid clones were selected from 30 clones in each strain, and 100 clones were obtained from 10 strains. The individuals of 100 diploid clones were used for DNA extraction.

#### DNA Extraction

*Artemia* genomic DNA used for ISSR analysis was isolated as described by Sun (1999) with some modifications: (1) after removing the digestive tract, each individual was directly immersed in a 200- $\mu$ L solution (100 mM L<sup>-1</sup> EDTA, 10 mM L<sup>-1</sup> Tris-HCl), containing 25  $\mu$ L 10% SDS and 10  $\mu$ L 20 mg mL<sup>-1</sup> proteinase K; (2) the incubated temperature was increased to 60°C; (3) non purified DNA was directly extracted and used to ISSR amplification. After isolation, DNA was stored in 10- $\mu$ L TE solution. DNA quality and quantity were determined by 0.8% agarose gel electrophoresis.

#### ISSR PCR Amplification

Twenty ISSR primers were synthesized from Saibaisheng Inc based on core repeats (Zietkiewicz et al. 1994), anchored either at the 5' or 3' end (Table 2). All ISSR primers were evaluated for their ability to produce polymorphic bands.

ISSR amplifications was performed by using the ISSR primers in a 20  $\mu$ L reaction volume containing 2.0  $\mu$ L  $\times$ 10 buffer, 1.25  $\mu$ L of 25 mM MgCl<sub>2</sub>, 2  $\mu$ L of 10 mM dNTP, 1.0  $\mu$ L of primer at 10

pM, 5–10 ng DNA template, and 0.5  $\mu$ L of *Taq* DNA polymerase (Takara Inc) and 20  $\mu$ L water volume. An initial 5 min denaturation at 94°C was followed by 45 cycles of 94°C denaturation for 30 s, 52°C annealing for 45 s, 72°C extension for 2.0 min. Amplification cycles were followed by a final 7 min extension at 72°C. Amplification was carried out with a PCR Express machine (ThermoHybaid, Needham Heights, MA). The size and quality of PCR products were determined on 2.0% agarose gels. Molecular weights were estimated using DL2000 DNA marker (Takara Inc).

#### Data Analysis

Data were scored in function of the presence (1) or absence (0) of every amplification product, and the data were entered into a data matrix. Based on data matrix of ISSR, Nei's (1978) genetic identity (*I*) (1978) and Nei's genetic distances (*D*) (1987) between geographical strains were analyzed. Measurements of diversity including gene diversity (*H*) at each locus; observed number of alleles (*N<sub>a</sub>*); effective number of alleles (*N<sub>e</sub>*) and Shannon information index (*SI*); gene differentiation (*G<sub>st</sub>*), according to McDermott and McDonald (1993), were estimated using the POPGENE 1.32 statistical package. Based on the matrix of genetic identity (Nei 1978) cluster analyses were performed using unweighted pair/group method with arithmetic averages (UPGMA) (Sneath & Sokal 1973). The dendrogram was constructed by software PHYLIP 3.5c neighbor and TreeView1.66.

## RESULTS

Using 20 ISSR primers we detected 170 bands of which 164 were polymorphic (96.47%). The level of polymorphism for each primer is quite variable, ranging from 54.12 per cent (in GH strain) to 55.10 per cent (87.06) (in PK strain). Band size ranged from 100–2200 bp. Representative ISSR fingerprints obtained with primer (AC)<sub>8</sub>T are shown in Figure 1.

#### Population Genetic Structure

Table 3 showed the number of polymorphic loci and percentage polymorphic loci, mean observed number of alleles (*N<sub>a</sub>*), mean effective number of alleles (*N<sub>e</sub>*), mean Nei's gene diversity (*H*) and mean Shannon's Information index (*I*) in the 100 clones from 10 Chinese *Artemia* parthenogenetic strains. The observed number of alleles (*N<sub>a</sub>*) ranged from 1.8706 (PK) to 1.5412 (GH), mean value of *N<sub>a</sub>* was 1.7571. Compared with *N<sub>a</sub>*, the *N<sub>e</sub>* values (effective number of alleles) were lower, which ranged from 1.2557 (GH) to 1.6219 (LN). The Nei's gene diversity (*H*) ranged from 0.1551 (GH) to 0.3476 (LN), mean Nei's gene diversity was

TABLE 2.  
List of primers used for ISSR amplification

Primer	Sequence (5'–3')	Primer	Sequence (5'–3')
ISSR-1	B*DB (TCC) <sub>5</sub>	ISSR-11	(AG) <sub>8</sub> TG
ISSR-2	(TCC) <sub>8</sub> RY*	ISSR-12	(TC) <sub>8</sub> C
ISSR-3	VBV (CA) <sub>8</sub>	ISSR-13	(TG) <sub>8</sub> G
ISSR-4	VDV (GT) <sub>8</sub>	ISSR-14	(CA) <sub>6</sub> R
ISSR-5	(AG) <sub>8</sub> T	ISSR-15	(CA) <sub>6</sub> RY
ISSR-6	HVH (TG) <sub>7</sub> T	ISSR-16	(GT) <sub>6</sub> YR
ISSR-7	(CT) <sub>8</sub> A	ISSR-17	(GT) <sub>6</sub> AY
ISSR-8	(AC) <sub>8</sub> T	ISSR-18	(ACTG) <sub>4</sub>
ISSR-9	(AC) <sub>8</sub> G	ISSR-19	(GACA) <sub>4</sub>
ISSR-10	(TG) <sub>8</sub> GT	ISSR-20	(CAC) <sub>6</sub>

\* Y = C T; R = A G; H = A C T; B = C G T; V = A C G; D = A G T.

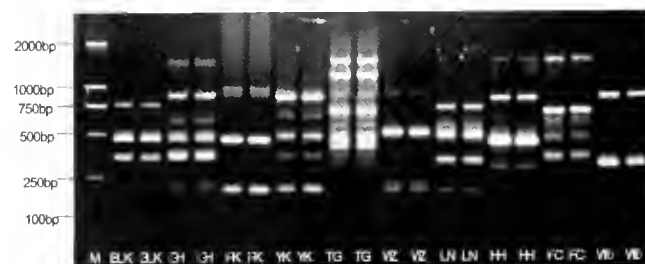


Figure 1. ISSR PCR fingerprints of reactions amplification of 10 parthenogenetic *Artemia* strains related genera using the ISSR-8 primer. The abbreviations of different geographic *Artemia* strains shown in Table 1. The lane marked M shows the 2-kb ladder being used as marker (DL-2000).

TABLE 3.  
Summary of population genetic structure for all loci

Strains	No. Clones	$N_a^*$	$N_e$	$H$	$SI$	No. Polymorphic Loci (percentage)
WD	10	1.7706 ± 0.4217	1.5532 ± 0.3756	0.3108 ± 0.1917	0.4530 ± 0.2680	131 (77.06%)
WZ	10	1.6353 ± 0.4828	1.3665 ± 0.3739	0.2156 ± 0.1964	0.3249 ± 0.2784	108 (63.53%)
FC	10	1.7412 ± 0.4393	1.4914 ± 0.3976	0.2768 ± 0.2014	0.4076 ± 0.2786	126 (74.12%)
TG	10	1.7882 ± 0.4098	1.5547 ± 0.3727	0.3124 ± 0.1893	0.4565 ± 0.2630	134 (78.82%)
HH	10	1.8294 ± 0.3773	1.6078 ± 0.3411	0.3421 ± 0.1734	0.4972 ± 0.2420	141 (82.94%)
LN	10	1.8471 ± 0.3610	1.6219 ± 0.3456	0.3476 ± 0.1720	0.5050 ± 0.2372	144 (84.71%)
YK	10	1.7588 ± 0.4291	1.5443 ± 0.3850	0.3041 ± 0.1977	0.4427 ± 0.2754	129 (75.83%)
PK	10	1.8706 ± 0.3366	1.6063 ± 0.3318	0.3443 ± 0.1645	0.5040 ± 0.2250	148 (87.06%)
BLK	10	1.7882 ± 0.4098	1.5556 ± 0.3510	0.3162 ± 0.1850	0.4616 ± 0.2603	134 (78.82%)
GH	10	1.5412 ± 0.4998	1.2557 ± 0.3350	0.1551 ± 0.1846	0.2398 ± 0.2645	92 (54.12%)
Mean	100	1.7571 ± 0.1000	1.5157 ± 0.1173	0.2925 ± 0.0620	0.4292 ± 0.0855	164 (96.47%)

\*  $N_a$  = Observed number of alleles;  $N_e$  = Effective number of alleles [Kimura and Crow (1964)];  $H$  = Nei's (1973) gene diversity;  $SI$  = Shannon's Information index [Lewontin (1972)]; [See Nei (1987) Molecular Evolutionary Genetics (p. 176–187)]

0.2925. Shannon's Information index ( $SI$ ) estimated a measure of intrapopulation diversity, the highest value (0.5050) was found in the LN strain and the lowest value was 0.2398 in the GH strain, mean value was 0.4292. The Nei's genetic identity ( $I$ ) and genetic distance ( $D$ ) are examined for all pairwise comparisons between the subpopulations (Table 4). The genetic distances for all comparisons range from 0.0352 (between HH and TG) to 0.3353 (between YK and GH). The Nei's genetic identity among these 10 *Artemia* parthenogenetic strains (subpopulations) range from 0.7151(YK/GH) to 0.9654(TG/HH). The UPGMA dendrogram of Nei's genetic identity for 10 Chinese *Artemia* parthenogenetic strains showed that different geographic parthenogenetic strains were divided into 3 groups (subpopulation): Liaoning Province; Shandong and Hebei Province and Qinghai and Xinjiang (Fig. 2).

#### Genetic Diversity and Genetic Differentiation

Table 5 shows the total variation ( $H_t$ ), the average variation within populations ( $H_s$ ) and gene differentiation ( $G_{st}$ ) for pairwise strains and mean value of  $H_t$ ,  $H_s$  and  $G_{st}$  of all 100 clones from 10 Chinese *Artemia* parthenogenetic strains. The highest  $H_t$  value (0.3902) is found between LN and BLK strains, and the lowest value is 0.2639 between WZ and FC strains. The mean value of  $H_t$  from 100 clones was 0.3895, indicating that about 38.95 percent of genetic variation among the different strains. The  $H_s$  value is variable, ranges from 0.3459 (LN/PK) to 0.1853 (WZ/GH), with

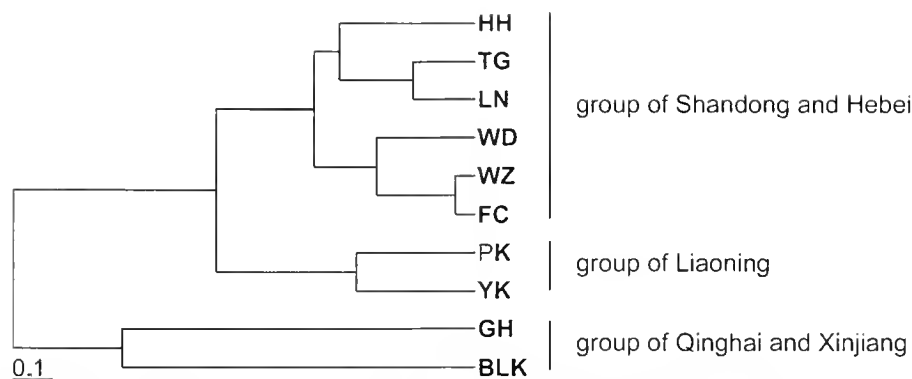
the mean value of  $H_s$  100 clones was 0.2925. The values of gene differentiation ( $G_{st}$ ) of 100 clones from 10 Chinese *Artemia* parthenogenetic strains range from 0.3464 (WZ/GH) to 0.0674 (WZ/FC), the mean value was 0.2492. The regression line based on  $\log_{10} G_{st}$  values and  $\log_{10} Km$  (geographical distance) pairwise among 10 subpopulations of *Artemia parthenogenetica*, is plotted by SPSS software. There is a significant correlation of  $t$ -test of regression coefficient ( $t = 7.7887$ ,  $P = 0.0001$ ). The regression equation is  $\text{Log}G_{st} = 0.2677\text{Log} Km - .5679$ ,  $R^2 = 0.5909$ .

#### DISCUSSION

ISSR has been successfully used to reveal genetic variation in silkworm (Pradeep et al. 2005), in aphids (Abbot, 2001) and in *Fenneropenaeus chinensis* shrimp (Wang & Kong, 2002), to characterize genome diversity (Yang et al. 1996), and to determine the origin of hybrids (Wolfe et al. 1998). The primers are anchored at their 3' end, to ensure that the annealing of the primer occurs only at the 3' or 5' end of the microsatellite motif, thus obviating internal priming and smear formation. The anchor also allows only a subset of the targeted inter-repeat regions to be amplified, thereby reducing the high number of PCR products expected from the priming of dinucleotide inter-repeat regions to a set of about 10–50 easily resolvable bands. Pattern complexity can be tailored by applying different primer lengths and sequences (Zietkiewicz et al. 1994). Based on its unique characters, the ISSR technique can

TABLE 4.  
Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

	WD	WZ	FC	TG	HH	LN	YK	PK	BLK	GH
WD	****	0.8940	0.9392	0.9015	0.9125	0.9112	0.8278	0.9034	0.8557	0.7637
WZ	0.1121	****	0.9536	0.9125	0.8996	0.8866	0.8422	0.8515	0.7614	0.7593
FC	0.0627	0.0475	****	0.9054	0.9023	0.8932	0.8573	0.8980	0.7976	0.7533
TG	0.1037	0.0915	0.0994	****	0.9654	0.9031	0.8599	0.8564	0.8039	0.7624
HH	0.0916	0.1058	0.1028	0.0352	****	0.9525	0.8466	0.8691	0.8302	0.7720
LN	0.0930	0.1204	0.1130	0.1019	0.0487	****	0.8352	0.8668	0.8257	0.7618
YK	0.1890	0.1717	0.1539	0.1509	0.1665	0.1801	****	0.9196	0.7822	0.7151
PK	0.1016	0.1608	0.1076	0.1550	0.1403	0.1430	0.0838	****	0.8342	0.7251
BLK	0.1559	0.2726	0.2262	0.2183	0.1860	0.1915	0.2456	0.1813	****	0.8266
GH	0.2695	0.2753	0.2833	0.2713	0.2587	0.2721	0.3353	0.3214	0.1904	****



**Figure 2.** UPGMA dendrogram of Nei's genetic identity (*I*) for Chinese parthenogenetic *Artemia* strains. The 10 Chinese *Artemia parthenogenetica* strains can be significant divided into three major groups (subpopulation): Liaoning (PK and YK); Shandong and Hebei (HH, TG, LN, WD, WZ and FC) and Qinghai and Xinjiang (GH and BLK). For abbreviations of the different geographic *Artemia* strains, see Table 1.

detect more genetic loci than isozyme and has higher stability than RAPD. This is the first report of using ISSR markers in surveying genetic structure and differentiation in *Artemia parthenogenetica*. ISSR fingerprints clearly distinguished all the tested *Artemia parthenogenetica* populations. The experimental results show high genetic diversity and difference among 10 different geographic strains (subpopulation) of *A. parthenogenetica* in China. There are high proportions (96.47%) of polymorphic loci, indicating the higher-level variation in the 10 different geographic strains. Nascetti et al. (2003) and Hou et al. (1993) by isozymes and Sun et al. (2000) by AFLP and RAPD found high levels of genetic diversity, high levels of genetic variability and high proportions of polymorphic loci in parthenogenetic populations, although different molecular marker techniques were used in the experiments the conclusions were the same. Based on our experimental data earlier, we supported their opinions of high-level genetic diversity and high proportion of polymorphic loci in *Artemia parthenogenetica* populations. Nascetti et al. (2003) reported high levels of heterozygosity of *A. parthenogenetica* populations from Italy arranged from 0.135–0.185 by isozymes. In this study, because of limitation of the ISSRs technique, heterozygosity of different geographic populations was not calculated.

The UPGMA dendrogram based on Nei's genetic identity for Chinese *Artemia parthenogenetica* strains showed that different geographic parthenogenetic strains can be divided into three groups (subpopulation): Liaoning Province, group of Shandong and Hebei Province; Qinghai Province and Xinjiang Autonomous Region. Moreover, compared with the inland saltlake group of Qinghai and Xinjiang, the coastal groups, which included the groups of the Liaoning and Shandong and Hebei Province have a closer relationship. In agreement, the AFLP marker (Sun 1999) showed that *A. parthenogenetica* from inland and coastal origin, group into two different clusters, and allozyme analysis (Gao et al. 1994) also indicate that there is a significant difference between the populations from coastal China (Huanghai, Hebei province, and Dalian, Liaoning province) and from inland salt lakes (Xinjiang Autonomous Region). Hence, the parthenogenetic populations from inland salt lakes could have followed an evolutionary path that is different from that of the coastal parthenogenetic populations, or the large genetic differences possibly occur because of geographic isolation. To explore the reason for relationships among different strains, we introduce the parameter of *Gst* (Nei & Chakraborty 1973). *Gst*, which can be used to explain the population genetic differentiation, is equivalent to *Fst* (Wright, 1951) when there are only two alleles at a locus, and, in the case of

multiple alleles; *Gst* is equivalent to the weighted average of *Fst* for all alleles (Nei & Chakraborty 1973). In this study, we did not find statistics data of *Gst* value in other strains, therefore *Gst* was compared with *Fst* of different geographic strains. The *Gst* value (0.3464) is higher within distant geographic subpopulations (WZ/BLK), average value is 0.2492, implying a higher genetic differentiation among populations. The *Gst* values (0.036–0.3464) of *A. parthenogenetica* in China is higher than that of the Chinese bisexual populations of *Artemia* (*Fst*, 0.0024–0.1297) (Xin et al. 2000), which revealed that high differentiation level among *A. parthenogenetica* within population (clones) (24.92% of variation within population and 75.08% of variations among populations of *A. parthenogenetica*). The adverse surroundings conditions in habitats of *Artemia* populations (shortage of food, higher salinity, irregular temperature) could result in higher levels of genetic diversity, differentiation and polymorphic phenomena in *A. parthenogenetica* populations, and selection plays an important role in the processes (Bowen et al. 1988, Browne & Hoopes 1990, Lenz & Browne 1991, Browne 1992, Hou et al. 1993, Nascetti et al. 2003). Meanwhile, the different environmental conditions could be responsible for selective fixation in heterozygosity of many loci and for high genetic divergence observed between either diploid/polyploidy or polyploidy populations (Barigozzi 1974, Nascetti et al. 2003). Once heterozygosity originates, it could maintain through selective pressure caused by environmental conditions (Zhang & King 1992). Although the earlier-mentioned hypothesis is supported by experimental results of Nascetti et al. (2003), Hou et al. (1993) and Zhang and King (1992) the formation mechanism of high levels of genetic diversity of *A. parthenogenetica* needs to be explored further.

Population genetic structure and *Gst* values can be changed by migration among individuals of different populations. *Artemia* cysts were suited for passive dispersal by wind, waterfowl or man (Persoone & Sorgeloos 1980), this passive migration may change gene diversity (*Gst* and *Fst*) and gene flow (*Nm*). In this study, the reasons for causing high-level genetic variations in population could be migration. We estimated the relationship *Gst* (intrapopulation genetic variations) and geographic distance (Fig. 3). Figure 3 shows, a clear tendency for higher *Gst* value with far geographical distance (*Km*) and revealed high genetic differentiations with far geographical distance. The Cause for high level genetic differentiation was that geographic distance plays an important role in *Artemia* cysts dispersal, cysts were difficult to disperse alone in long distances. The genetic differentiation (*Gst*) levels among these subpopulations raise with the increasing geographical dis-

TABLE 5.  
Nei's analysis of gene diversity in subpopulations

Strains	No. clones	<i>H<sub>i</sub></i>	<i>H<sub>s</sub></i>	<i>G<sub>st</sub></i>
WD-FC	20	0.3153 ± 0.0388	0.2938 ± 0.0340	0.0684
WD-TG	20	0.3455 ± 0.0279	0.3116 ± 0.0258	0.0982
WD-WZ	20	0.3029 ± 0.0355	0.2632 ± 0.0285	0.1312
WD-HH	20	0.3560 ± 0.0260	0.3264 ± 0.0245	0.0830
WD-LN	20	0.3591 ± 0.0246	0.3292 ± 0.0235	0.0833
WD-YK	20	0.3670 ± 0.0224	0.3074 ± 0.0219	0.1624
WD-BLK	20	0.3630 ± 0.0235	0.3135 ± 0.0226	0.1365
WD-GH	20	0.3251 ± 0.0293	0.2329 ± 0.0196	0.2834
WZ-FC	20	0.2639 ± 0.0349	0.2462 ± 0.0319	0.0674
WZ-TG	20	0.2968 ± 0.0333	0.2640 ± 0.0285	0.1109
WZ-HH	20	0.3163 ± 0.0288	0.2788 ± 0.0246	0.1184
WZ-LN	20	0.3237 ± 0.0293	0.2816 ± 0.0249	0.1300
WZ-YK	20	0.3188 ± 0.0267	0.2598 ± 0.0208	0.1849
WZ-PK	20	0.3346 ± 0.0239	0.2799 ± 0.0195	0.1635
WZ-BLK	20	0.3541 ± 0.0262	0.2659 ± 0.0217	0.2492
WZ-GH	20	0.2836 ± 0.0392	0.1853 ± 0.0194	0.3464
FC-TG	20	0.3280 ± 0.0309	0.2946 ± 0.0276	0.1021
FC-HH	20	0.3435 ± 0.0283	0.3094 ± 0.0254	0.0992
FC-LN	20	0.3493 ± 0.0276	0.3122 ± 0.0248	0.1063
FC-YK	20	0.3411 ± 0.0252	0.2904 ± 0.0219	0.1486
FC-PK	20	0.3461 ± 0.0263	0.3105 ± 0.0235	0.1027
FC-BLK	20	0.3678 ± 0.0271	0.2965 ± 0.0240	0.1939
FC-GH	20	0.3135 ± 0.0331	0.2159 ± 0.0187	0.3113
TG-HH	20	0.3390 ± 0.0298	0.3272 ± 0.0286	0.0346
TG-LN	20	0.3625 ± 0.0255	0.3300 ± 0.0238	0.0898
TG-YK	20	0.3567 ± 0.0246	0.3082 ± 0.0225	0.1359
TG-PK	20	0.3766 ± 0.0199	0.3283 ± 0.0192	0.1283
TG-BLK	20	0.3815 ± 0.0199	0.3143 ± 0.0204	0.1763
TG-GH	20	0.3263 ± 0.0306	0.2337 ± 0.0207	0.2837
HH-LN	20	0.3604 ± 0.0268	0.3449 ± 0.0253	0.0432
HH-YK	20	0.3751 ± 0.0214	0.3231 ± 0.0210	0.1387
HH-PK	20	0.3862 ± 0.0185	0.3432 ± 0.0181	0.1113
HH-BLK	20	0.3861 ± 0.0172	0.3291 ± 0.0180	0.1476
HH-GH	20	0.3365 ± 0.0256	0.2486 ± 0.0182	0.2612
LN-YK	20	0.3815 ± 0.0200	0.3258 ± 0.0201	0.1460
LN-PK	20	0.3895 ± 0.0172	0.3459 ± 0.0172	0.1118
LN-BLK	20	0.3902 ± 0.0165	0.3319 ± 0.0176	0.1494
LN-GH	20	0.3429 ± 0.0247	0.2513 ± 0.0169	0.2669
YK-PK	20	0.3515 ± 0.0227	0.3242 ± 0.0220	0.0777
YK-BLK	20	0.3853 ± 0.0176	0.3101 ± 0.0197	0.1950
YK-GH	20	0.3406 ± 0.0281	0.2296 ± 0.0198	0.3260
PK-BLK	20	0.3858 ± 0.0182	0.3302 ± 0.0183	0.1441
PK-GH	20	0.3550 ± 0.0235	0.2497 ± 0.0173	0.2966
BLK-GH	20	0.3037 ± 0.0279	0.2356 ± 0.0193	0.2240
Mean*	100	0.3895 ± 0.0159	0.2925 ± 0.0125	0.2492

The number of polymorphic loci is : 164

The percentage of polymorphic loci is : 96.47

Mean values of *H<sub>i</sub>*, *H<sub>s</sub>* and *G<sub>st</sub>* of 100 clones from 10 chinese *Artemia parthenogenetica* populations [See Nei (1987) Molecular Evolutionary Genetics (p. 187–192)]

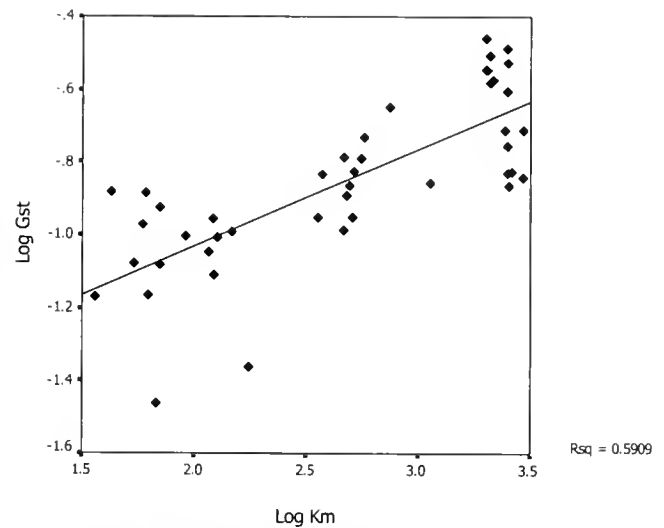


Figure 3. Regression line based on  $\log_{10} G_{st}$  values and  $\log_{10} Km$  (geographical distance) pairwise between 10 subpopulations of *Artemia parthenogenetica*. There is a significant correlation of *t*-test of regression coefficient ( $t = 7.7887$ ,  $P = 0.0001$ ). The regression equation is  $\text{Log}G_{st} = 0.2677\text{Log} Km - 1.5679$ ,  $R^2 = 0.5909$

tance, therefore the high *G<sub>st</sub>* levels may be results of genetic isolation by a geographical distance barrier. Conversely, WZ, FC and WD have a short geographic distance, and individuals of different populations may exchange very frequently by migration. *G<sub>st</sub>* values of WZ, FC and WD were low, which indicates the presence of low level genetic differentiations among these subpopulations. Although the gene flow could not be estimated among *Artemia parthenogenetica* strains, genetic differentiations among different strains may be affected by a geographical isolation barrier and migrating by birds, wind, workers of salt works and others (Andy et al. 2005; Browne et al. 1993).

In recent years, a large amount of male individuals (40% and above) were found in *A. parthenogenetica* from the Chinese coastal salt works, it was believed that this was caused by ecological invasion of *A. franciscana* or *A. sinica*, because cysts of *A. franciscana* or *A. sinica* were introduced into these salterns for aquaculture. The samples in this study were collected before ecological invasion occurred, so it is important to know basal data of the population genetic structure and clone diversity of *A. parthenogenetica* from China. Taxonomic status of invader and population genetic structure need to be studied further.

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## THE GROWTH OF JUVENILE CHINESE SHRIMP, *FENNEROPENAEUS CHINENSIS* OSBECK, AT CONSTANT AND DIEL FLUCTUATING TEMPERATURES

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**ABSTRACT:** The growth, food consumption, food conversion efficiency and apparent digestibility coefficient of juvenile Chinese shrimp, *Fenneropenaeus chinensis* Osbeck (body weight  $0.36 \pm 0.04$  g) were examined under different temperature regimes. The animals were subjected to 6 constant temperatures of 18°C, 22°C, 25°C, 28°C, 31°C and 34°C and diel fluctuating temperatures of  $22 \pm 2$ °C,  $25 \pm 2$ °C,  $28 \pm 2$ °C and  $31 \pm 2$ °C. The results showed that the growth rate of Chinese shrimp at the constant temperature regimes increased from 18°C to 31°C, whereas decreased significantly at 34°C. The growth rates of shrimp at  $22 \pm 2$ °C,  $25 \pm 2$ °C and  $28 \pm 2$ °C were significantly greater than those at the corresponding constant temperatures of 22°C, 25°C and 28°C, respectively, whereas no significant difference was found between  $31 \pm 2$ °C and 31°C. As compared with those at constant temperature, the mean temperature at which maximum growth rate of shrimp occurred shifted to cooler temperature at diel fluctuating temperatures. The shrimp consumed more food at  $25 \pm 2$ °C,  $28 \pm 2$ °C and  $31 \pm 2$ °C than those at 25°C, 28°C and 31°C, respectively, but no significant difference in apparent digestibility coefficient was found between the fluctuating and corresponding constant temperatures. The food conversion efficiency and energy assimilated into growth as percentage of energy from food for shrimp at  $22 \pm 2$ °C,  $25 \pm 2$ °C and  $28 \pm 2$ °C were significantly higher than at the corresponding constant temperatures. Therefore, more food consumption, high food conversion efficiency and more energy partitioned into growth might contribute to the enhanced growth rate at the fluctuating temperatures.

**KEY WORDS:** constant temperatures, diel fluctuating temperatures, growth, energy budget, *Fenneropenaeus chinensis*, shrimp

### INTRODUCTION

Temperature is one of the major physical factors influencing the growth rates of ectotherms. It is well known that the temperatures in natural aquatic systems fluctuate diurnally and seasonally. Most previous studies on the effects of temperatures on the growth of aquatic animals usually have been conducted under constant temperature regimes with an implicit assumption that growth in fluctuating temperatures would be approximated by growth at a constant temperature coinciding with the average temperature of the fluctuation. These studies have led to much insight into the influence of temperature on the growth of aquatic animals, however, the growth response of animals subjected to a fluctuating thermal environment, typical of those normally encountered in nature, would not been examined (Hokanson et al. 1977, Cox & Coutant 1981). For example, the use of average temperature to characterize growth in fluctuating temperature regimes had been questioned by Hokanson et al. (1977).

Until now, many studies have been conducted to investigate the effects of fluctuating temperatures on a variety of aquatic ectotherms, such as zooplankton (Halbach 1973, Van As et al., 1980), bivalves (Widdows 1976, Pilditch & Grant 1999) and crustaceans (Dame & Vernberg 1978, Miao & Tu 1993, Miao & Tu 1996), and most of them focused on fishes (Biette & Geen 1980, Cox & Coutant 1981, Diana 1984, Konstantinov et al. 1989, Lyytikäinen & Jobling 1998, Lyytikäinen & Jobling 1999, Sierra et al. 1999, Zdanovich 1999, Baras et al. 2000). Because of the differences in species and thermal regimes, the results from different studies were very inconsistent.

Chinese shrimp *Fenneropenaeus chinensis* (Osbeck 1765) is the most important marine species cultured in China, comprising approximately 80% of the total shrimp production. Chinese shrimp are mainly distributed in the Yellow Sea and usually migrate for reproduction and overwintering twice a year in Bohai Sea, Yellow

Sea and East China Sea (Ge & Wang 1995, Miao & Tu 1995). Thus, not only Chinese shrimp have experienced temperatures diurnally and seasonally fluctuations, but also they normally are exposed to varying temperatures when they move in water masses, vertically and horizontally, during feeding, swimming or predator avoidance. Until now, although a lot of studies on the effects of temperatures on the growth of this species have been conducted, most of them have focused on the effects of constant temperatures (Zhang et al. 1983, Wang et al. 1984, Miao & Tu 1995, Zhang et al. 1998), and only a few work dealing with the effect of fluctuating temperature on the growth of shrimp (Miao & Tu 1996) was reported.

In a previous study, it was found that the oxygen consumption of Chinese shrimp at a fluctuating temperature of  $27 \pm 3$ °C was significantly lower than that at a constant temperature of 27°C (Tian et al. 2004b), whereas the growth rate of shrimp at  $27 \pm 3$ °C was significantly higher than at the corresponding constant temperature of 27°C (Tian 2001), which implicated that there could be a bioenergetic mechanism involved in this difference between fluctuating thermal regimen and corresponding constant temperature. Based on this assumption, this study was designed to evaluate the growth rate, food consumption (FC), food conversion efficiency and energy budget of Chinese shrimp at diel fluctuating temperatures and constant temperature. The physiological and bioenergetic mechanisms involved in the optimization of shrimp growth in fluctuating thermal regimes were also investigated.

### MATERIALS AND METHODS

#### *Experimental Shrimp and Acclimation*

Chinese shrimp juveniles were obtained from the pond of the Hongdao Shrimp Farm, Qingdao, P.R. China. The shrimp were cultured in aerated fiberglass tanks with seawater and maintained at about 25°C for at least 3 days. Then the shrimp were acclimated to various constant temperature regimes, 18°C, 22°C, 25°C, 28°C, 31°C and 34°C at 1.5°C per day. When the desired tem-

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perature was reached, the shrimp were further kept in the target temperature for three days before the experiment started. During acclimation, the shrimp were fed twice daily to satiation with a commercial pellet manufactured by the Mawei Fishery Feed Co. Ltd., Fujian, China ( $43.39 \pm 0.22\%$  crude protein,  $9.74 \pm 0.30\%$  lipid,  $9.91 \pm 0.05\%$  ash,  $16.88 \pm 0.11\%$  energy and  $8.41 \pm 0.06\%$  moisture). A 14 h light: 10 h dark photoperiod was maintained.

#### Experimental Design and Facility

The thermal treatments included 6 constant temperatures, 18°C, 22°C, 25°C, 28°C, 31°C and 34°C, and four diel temperature fluctuations with daily means between 22 and 31. There were four replicates for each thermal treatment. In the fluctuating temperature treatments, the pattern of diel temperature fluctuations imitated the daily natural rhythm of field water temperature at the site of the present experiment ( $36^{\circ}1'N$ ,  $120^{\circ}3'E$ ) and amplitude of temperature fluctuation was set at  $\pm 2^{\circ}C$ . The minimum temperature was set at 0600 h, and increased gradually to the maximum at 1400 h, then was decreased to the minimum again at 0600 h next day.

During the experiment, the ambient temperature was controlled at  $16 \pm 0.5^{\circ}C$ . Four glass aquaria ( $45 \times 25 \times 30$  cm) were incubated in a water bath tank ( $170 \times 75 \times 30$  cm) and the temperature of aquaria was controlled by water bath. Each aquarium was covered with a 5-mm screen to prevent the shrimp jumping out of the aquaria. Two recirculating pumps (35 W) were applied in each water bath tank to ensure the even distribution of water temperature in whole tank. Two systems were applied to control different water thermal regimes. The constant temperature was maintained by the thermostatic regulation of immersion heaters (WMZK-01). The actual temperature was daily calibrated with a mercury thermal meter to the nearest  $0.1^{\circ}C$ . In the treatment with fluctuating temperatures, each designated temperature range was controlled by a heating and cooling coil.

#### Experimental Procedure and Management

After thermal acclimation, 20 shrimp were randomly chosen, weighed individually and stocked into four aquaria for corresponding constant temperature treatment with each aquarium holding five individuals after 24-h feed deprivation. The same procedure was applied to choose shrimp from 22°C, 25°C, 28°C and 31°C thermal acclimation tanks and stock into aquaria for the diel temperature fluctuations treatments, i.e.,  $22 \pm 2^{\circ}C$ ,  $25 \pm 2^{\circ}C$ ,  $28 \pm 2^{\circ}C$  and  $31 \pm 2^{\circ}C$ , respectively. There were four aquaria for each thermal treatment. Ten shrimp were randomly sampled from each corresponding thermal acclimation tank for measurement of initial proximate body composition. During the experiment, the shrimp were fed twice daily to satiation (at 0600 and 1800 h) with commercial pellets. The uneaten feed and feces were separately collected using a siphon tube within 1.5 h after feeding. The collected uneaten feed and feces were settled in a beaker, and then the water above was removed carefully. The exuviae (molted exoskeletons) were collected at times. The uneaten feed, feces and exuviae were dried at  $65^{\circ}C$ , respectively, and kept for further analysis. FC was estimated from the difference between the amount of food applied into the aquarium and food uneaten. At the end of the experiment, all the test shrimp were collected after 24-h starvation and dried at  $65^{\circ}C$  for 48 h.

The experiment was conducted from May 30, 2000 to July 2, 2000. During the experiment, water exchanges were made to all

treatments at the same time. Aeration was provided continuously and one-half to two-thirds of the water volume was exchanged every other day to ensure suitable water quality. Seawater used in the experiment was filtered by composite sand filter. During the course of the experiment, dissolved oxygen was maintained above 6.0 mg/L, the pH was around 7.8, ammonia was less than 0.24 mg/L, salinity of seawater was within 28–30 ppt, and a simulated natural photoperiod (14 L:10 D light/ darkness) was maintained.

#### Determination of Energy Contents and Estimation of Energy Budget

The energy contents of the whole shrimp, feed and feces were measured by the Parr 1281 Oxygen Bomb Calorimeter. The energy budget was calculated as the following equation for the crustacean energy budget (Petrusewicz & Macfadyen 1970):

$$C = G + F + U + E + R$$

where,  $C$  is the energy consumed in food;  $G$ , the energy deposited for growth;  $F$ , the energy lost in feces;  $U$ , the energy in excretion;  $E$ , the energy spent for exuvia, and  $R$ , the energy for respiration.

The estimation of  $U$  was based on the nitrogen budget equation (Levine & Sulkin 1979, Lemos & Phan 2001):

$$U = (C_N - G_N - F_N - E_N) \times 24,830$$

where  $C_N$  is the nitrogen consumed from food;  $F_N$ , the nitrogen lost in feces;  $G_N$ , the nitrogen deposited in shrimp body;  $E_N$ , the nitrogen lost in molting; 24,830, the constant of energy content in excreted nitrogen (J/g). The nitrogen contents in the formulated feed, shrimp, feces and molting shell were determined by the Kjeldahl method.

The value of  $R$  was calculated as the following energy budget equation:

$$R = C - G - F - U - E$$

#### Data Calculation and Statistical Analysis

All indices were calculated as follows: specific growth rate (SGR, %/d) =  $100 \times (\ln W_t - \ln W_0) / t$ ; food conversion efficiency (FCE, %) =  $100 \times (W_t - W_0) / C$ ; apparent digestibility coefficient (ADC, %) =  $100 \times (C - F) / C$ , where,  $W_t$  and  $W_0$  are the final and initial weights (g),  $t$  is the feeding duration (d),  $C$  is daily FC (g), and  $F$  is the daily fecal production (g).

Experimental data expressed as aquarium means were analyzed using SPSS 10.0 (SPSS Inc., Richmond, CA, USA), with possible differences among data being tested by the student's  $t$ -test and one-way ANOVA. Duncan multiple range test was used to test the differences between treatments.  $P < 0.05$  was accepted as the level of statistical significance. Log transformations were used to homogenize the variance of body weight before data analyses. Arcsine transformations were used for feeding rate, FCE and apparent digestion rate to normalize the data distribution.

#### RESULTS

The growth rate of Chinese shrimp at constant temperatures increased with the temperatures from 18°C to 31°C, whereas it decreased significantly at 34°C (Table 1 and Fig. 1). Referred to studies of Miao and Tu (1993), the relationship between constant temperature ( $T, ^{\circ}C$ ) and SGR of Chinese shrimp was best described by polynomial regression analysis:

$$\begin{aligned} \text{SGR} &= -0.0028T^3 + 0.1976T^2 - 4.3902T + 32.4816 \\ R^2 &= 0.8217 \quad F = 39.90 \quad n = 24 \end{aligned}$$

TABLE 1.  
Growth of Chinese shrimp at constant and fluctuating temperatures (mean  $\pm$  SE)

Treatments	Temperature (°C)	No. Shrimp	Initial Weight (g)	Final Weight (g)	Duration (d)	Daily Increment (mg/d.ind)
CT18	18	20	0.35 $\pm$ 0.02	0.52 $\pm$ 0.07	33	5.34 $\pm$ 2.08
CT22	22	20	0.37 $\pm$ 0.01	0.67 $\pm$ 0.06	33	9.10 $\pm$ 0.16
CT25	25	20	0.35 $\pm$ 0.02	0.86 $\pm$ 0.09	33	15.54 $\pm$ 1.85
CT28	28	20	0.35 $\pm$ 0.02	0.96 $\pm$ 0.08	33	18.67 $\pm$ 3.23
CT31	31	20	0.36 $\pm$ 0.02	1.07 $\pm$ 0.08	33	21.43 $\pm$ 0.70
CT34	34	20	0.33 $\pm$ 0.04	0.63 $\pm$ 0.05	33	9.10 $\pm$ 2.08
FT22	22 $\pm$ 2	20	0.35 $\pm$ 0.03	0.81 $\pm$ 0.04	33	13.94 $\pm$ 1.12
FT25	25 $\pm$ 2	20	0.35 $\pm$ 0.06	1.09 $\pm$ 0.08	33	22.42 $\pm$ 1.62
FT29	28 $\pm$ 2	20	0.34 $\pm$ 0.02	1.32 $\pm$ 0.07	33	29.70 $\pm$ 1.67
FT31	31 $\pm$ 2	20	0.35 $\pm$ 0.01	1.23 $\pm$ 0.06	33	26.67 $\pm$ 0.51

CT, constant temperature; FT, fluctuating temperature.

The shrimp exhibited different growth trends at diel fluctuating temperatures compared with those at constant temperature regimen (Table 1 and Fig. 1). The daily increment and SGR of shrimp at diel fluctuating temperatures changed from 13.94 mg per day and 2.54% per day to 29.70 mg per day and 4.11% per day, respectively. The growth rates of shrimp at 22  $\pm$  2°C, 25  $\pm$  2°C and 28  $\pm$  2°C were significantly greater than those at the corresponding constant temperatures of 22°C, 25°C and 28°C, respectively, whereas no significant difference was found between 31°C  $\pm$  2°C and 31°C ( $P < 0.05$ ). The best growth occurred at 28°C  $\pm$  2°C under fluctuating temperature regimen, whereas best growth occurred at 31°C under constant temperature regimen. Thus, from the growth curves of shrimp, it can be seen that the mean temperature at which maximum growth rate of shrimp occurred shifted to cooler temperature at diel fluctuating temperatures as compared with those at constant temperature.

Compared with corresponding constant temperature, FC of shrimp at 25  $\pm$  2°C, 28  $\pm$  2°C and 31  $\pm$  2°C were significantly higher than at corresponding constant temperatures ( $P < 0.05$ ); however, no significant difference was found between 22  $\pm$  2°C and 22°C ( $P > 0.05$ ) (Fig. 2).

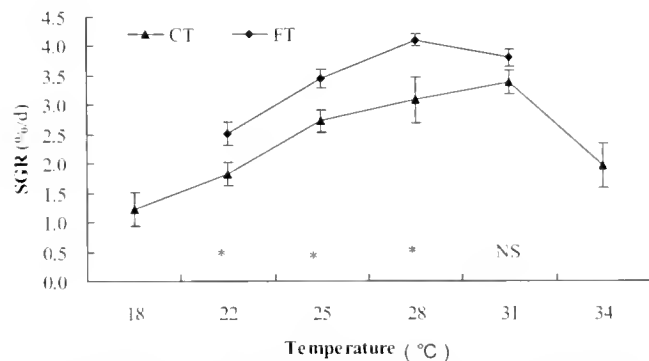


Figure 1. SGR of Chinese shrimp at constant and fluctuating temperatures. Each point is the mean  $\pm$  SE of determinations from four replicates, error bars represent 1 SE. NS means no significantly difference between the daily mean values of oxygen consumption of shrimp for the two patterns of fluctuating temperature regimen and control one and \* means the significant difference ( $P < 0.05$ ). CT, constant temperature; FT, fluctuating temperature.

There was no difference in ADC between diel fluctuating temperature and corresponding constant temperature ( $P > 0.05$ , Fig. 3).

Food conversion efficiencies of shrimp at fluctuating temperatures of 22  $\pm$  2°C, 25  $\pm$  2°C and 28  $\pm$  2°C were higher than at the corresponding constant temperatures of 22°C, 25°C and 28°C ( $P < 0.05$ ), respectively, but there was no significant difference between 31°C and 31  $\pm$  2°C ( $P > 0.05$ ) (Fig. 4).

Table 2 shows the patterns of energy allocation in the test shrimp at fluctuating temperatures and corresponding constant temperatures. The shrimp at the fluctuating temperatures of 22  $\pm$  2°C, 25  $\pm$  2°C and 28  $\pm$  2°C assimilated more energy for growth than those at constant temperatures of 22°C, 25°C and 28°C ( $P < 0.05$ ), respectively, whereas no significant difference was found between 31  $\pm$  2°C and 31°C ( $P > 0.05$ ). The shrimp at 28  $\pm$  2°C spent less energy in respiration than those at 28°C ( $P < 0.05$ ). No differences were found between the fluctuating and corresponding constant temperatures in the allocation of consumed energy to feces, excretion and exuvia.

## DISCUSSION

The positive influence of fluctuating temperatures on the growth of crustaceans has been reported in cladocerans, copepods, crabs and penaeid shrimp. The development and growth of *Daph-*

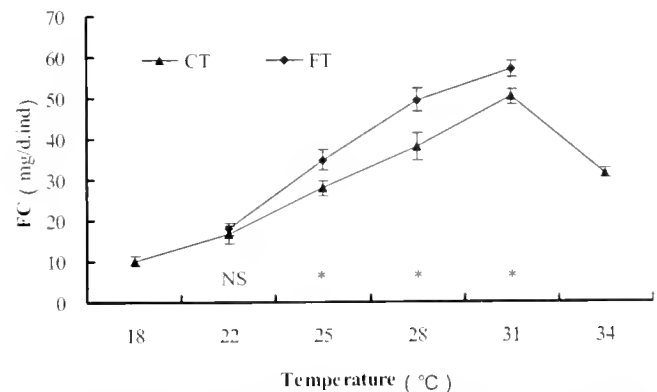


Figure 2. FC rate of Chinese shrimp at constant and fluctuating temperatures. For treatment abbreviations and symbols, refer to Fig. 2.

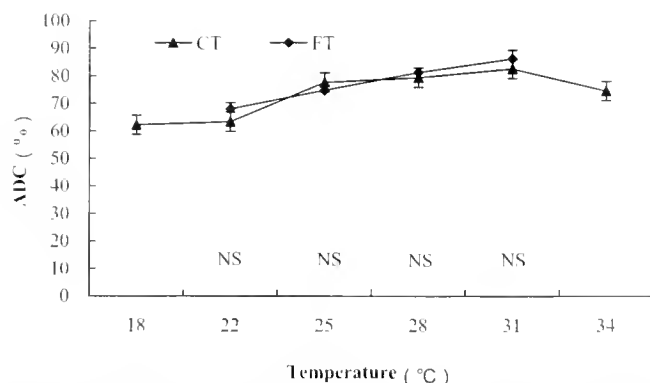


Figure 3. ADC of Chinese shrimp at constant and fluctuating temperatures. For treatment abbreviations and symbols, refer to Fig. 2.

*nia pulex* (De Geer), *Pseudocalanus minutus* (Kroeyer), mud-crab *Rhithropanopeus harrissi* (Gould), Chinese mitten crab *Eriocheir sinensis* (H. Milne Edwards), redbtail shrimp *F. penicillatus* (Alcock) and Chinese shrimp increased at moderate diel fluctuating thermal regimes (Lock & McLaren 1970, Costlow & Bookhout 1971, Van As et al. 1980, Miao & Tu 1993, Miao & Tu 1996, Wang 1999). In this study, the shrimp at fluctuating temperatures of  $22 \pm 2^\circ\text{C}$ ,  $25 \pm 2^\circ\text{C}$  and  $28 \pm 2^\circ\text{C}$  exhibited higher growth rate than those at corresponding constant temperatures of  $22^\circ\text{C}$ ,  $25^\circ\text{C}$  and  $28^\circ\text{C}$ , and better growth occurred at  $28^\circ\text{C} \pm 2^\circ\text{C}$ . In comparison, the shrimp at  $31^\circ\text{C}$  grew best under constant temperature regimes. Thus, the mean temperature at which maximum growth rate of shrimp occurred shifted to cooler temperature at diel fluctuating temperatures as compared with those at constant temperature, which is similar to results of Hokanson et al. (1977) and Lyytikäinen and Jobling (1999) in rainbow trout *Salmo gairdneri* (Richardson) and Arctic charr *Salvelinus alpinus* (Linnaeus). The growth rate of shrimp appeared to be accelerated at fluctuating temperatures whose mean temperatures are below the corresponding constant optimum temperature for growth, whereas it retarded at fluctuating thermal regimes whose mean temperatures are above the constant optimum temperature for growth. This result indicated that the diel fluctuations within the range normally selected by shrimp (i.e., physiological optimum and lower) were beneficial to growth. Fluctuations at the mean temperatures above the physiological optimum, however, might inhibit the growth of shrimp.

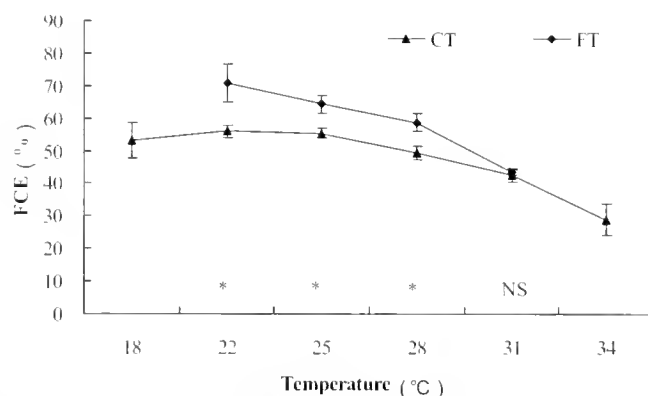


Figure 4. FCE of Chinese shrimp at constant and fluctuating temperatures. For treatment abbreviations and symbols, refer to Fig. 2.

The mechanisms for the enhancement in growth of crustacean at diel fluctuating temperatures were still investigated less thoroughly. Dame and Vernberg (1978) showed that diel fluctuating temperature regimes significantly depressed oxygen consumption in the  $15^\circ\text{C}$  to  $25^\circ\text{C}$  temperature range in the mud crab *Panopeus herbstii* (H. Milne-Edwards) and the fiddler crab *Uca pugilator* (Bosc) when compared with rates of animals subjected to constant acclimation rates. Because this depression of metabolic rates occurs over that portion of the yearly temperature range within which the animals are most active, it is suggested that these organisms use energy more efficiently when subjected to natural cyclic temperature conditions than when subjected to constant temperature environments. Tian et al. (2004b) also obtained the similar result in Chinese shrimp. The oxygen consumption of Chinese shrimp at a fluctuating temperature of  $27^\circ\text{C} \pm 3^\circ\text{C}$  was significantly lower than that at a constant temperature of  $27^\circ\text{C}$ , which could explain the increased growth at same fluctuating temperatures partially (Tian 2001). In this study, increased FC were found in shrimp at fluctuating temperatures of  $25^\circ\text{C} \pm 2^\circ\text{C}$  and  $28^\circ\text{C} \pm 2^\circ\text{C}$ , and better FCE occurred at  $22 \pm 2^\circ\text{C}$ ,  $25 \pm 2^\circ\text{C}$  and  $28 \pm 2^\circ\text{C}$ , whereas there was no difference in ADC between diel fluctuating temperature and corresponding constant temperature. The study on energy allocation showed that the shrimp at fluctuating temperature of  $22 \pm 2^\circ\text{C}$ ,  $25 \pm 2^\circ\text{C}$  and  $28 \pm 2^\circ\text{C}$  partitioned more energy for growth than those at constant temperatures of  $22^\circ\text{C}$ ,  $25^\circ\text{C}$  and  $28^\circ\text{C}$ , respectively. Thus, the enhancement in the growth of shrimp in the present study could mainly be ascribed to energetic

TABLE 2.

Allocation of the consumed energy in Chinese shrimp at constant and fluctuating temperatures (mean  $\pm$  SE)

Treatments	G/C (%)	F/C (%)	U/C (%)	E/C (%)	R/C (%)
CT22	13.94 $\pm$ 1.34 <sup>a</sup>	15.59 $\pm$ 1.12	6.81 $\pm$ 0.15	0.80 $\pm$ 0.09	62.86 $\pm$ 3.12
FT22	18.16 $\pm$ 1.12 <sup>b</sup>	14.81 $\pm$ 1.23	6.05 $\pm$ 1.02	0.63 $\pm$ 0.04	60.35 $\pm$ 2.41
CT25	13.82 $\pm$ 0.48 <sup>a</sup>	12.04 $\pm$ 0.77	7.06 $\pm$ 0.26	0.44 $\pm$ 0.15	66.64 $\pm$ 1.52
FT25	15.62 $\pm$ 0.26 <sup>b</sup>	13.56 $\pm$ 1.02	6.47 $\pm$ 0.28	0.28 $\pm$ 0.03	64.06 $\pm$ 1.91
CT28	12.35 $\pm$ 1.56 <sup>a</sup>	11.73 $\pm$ 1.04	7.03 $\pm$ 0.27	0.51 $\pm$ 0.11	68.38 $\pm$ 1.44 <sup>a</sup>
FT28	17.97 $\pm$ 1.58 <sup>b</sup>	10.43 $\pm$ 0.96	6.41 $\pm$ 0.20	0.46 $\pm$ 0.11	64.72 $\pm$ 0.55 <sup>b</sup>
CT31	12.14 $\pm$ 0.57	8.41 $\pm$ 0.45	7.61 $\pm$ 0.06	0.52 $\pm$ 0.01	71.32 $\pm$ 0.41
FT31	12.66 $\pm$ 0.21	6.75 $\pm$ 1.54	7.80 $\pm$ 0.12	0.41 $\pm$ 0.01	72.38 $\pm$ 1.31

CT, constant temperature; FT, fluctuating temperature; R/C, energy for respiration/energy consumed in food; G/C, energy for growth/energy consumed in food; E/C, energy for exuvia/energy consumed in food; U/C, energy for excretion/energy consumed in food; F/C, energy for faeces/energy consumed in food. Means with different letters were significantly different from each other ( $P < 0.05$ ).

advantage at optimal diel fluctuating temperatures, high FCE, reduced metabolism and more energy partitioned into growth although more FC might be one of mechanisms for increased growth as well.

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## DISTRIBUTION AND ABUNDANCE OF PELAGIC SHRIMPS FROM THE DEEP SCATTERING LAYER OF THE EASTERN ARABIAN SEA

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**ABSTRACT** The sonic scattering layer (SSL) or deep scattering layer (DSL) of the eastern Arabian Sea of India is found in depths between surfaces to 750 m with varying thicknesses and in multiple layers. There is a distinct resident community of pelagic shrimps that is widely distributed in the DSL biocomposition. Pelagic shrimps formed 19% of the total biomass and 47% of the micronekton biomass of the DSL. Many of them serve as important food resources to several pelagic/mesopelagic organisms. Pelagic shrimps concentrate in the deep scattering layer and diurnally migrate in the water column. The estimated average abundance pelagic shrimps ranged between 0.04–106 /1000m<sup>3</sup>. Shrimp catches were higher during night at 50–200 m depth than day. The dominant families encountered in the catches were Thalassocarididae, Pasiphaeidae, Sergestidae and Oplophoridae. Dominant genera of pelagic shrimps present were *Gemmadus*, *Sergestes*, *Sergia*, *Lucifer*, *Oplophorus*, *Acanthephyra*, *Nematocarcinus*, *Leptochela*, and *Thalassocaris*. Species *Sergestes seminudus*, *Thalassocaris crinata* and *Leptochela robusta* were present in swarms.

**KEY WORDS:** pelagic shrimps, deep scattering layer, geographical distribution, vertical migration, swarming behavior

### INTRODUCTION

Since the challenger expedition (1873–1876), the occurrence of bright red pelagic shrimps in the mid and deep waters of the ocean has been known. Recently, much attention has been paid to discover their role in the productivity of the ocean. The importance of pelagic shrimps as sonic scatters, food for larger animals, and as agents in energy transfer to the deep sea is well documented (Omori 1974, Kikuchi & Omori 1985). Marine organisms aggregate at specific depths in the ocean and the scattered sound waves from these organisms can be recorded as a scattering layer on the echogram of an echo sounder. This layer is referred to as sound-scattering layer (SSL) or deep scattering layer (DSL) and has been observed in all the oceans (Sameoto et al. 1985, Iida et al. 1996). Hays (2003) stated that the DSL organisms, which ascend around dusk and descend around dawn presumably reflecting the predator-prey tracking. Often discrete layers are evident at different depths, each layer composed of different species or developmental stages (Tarling et al. 2001). The DSL is a layer of living organisms, ranging from almost microscopic zooplanktons like copepods to macroorganisms like shrimps, squids and fishes that prey from within and outside the DSL (Ingmanson & William 1973). According to Omori (1974), out of a total of about 2,000 species of shrimps recorded from the world oceans, as many as 210 species pass their complete life in the pelagic realm. In Indian waters, the earliest studying on the taxonomy of pelagic shrimps was that of Alcock (1901) who listed several species along with benthic forms from the collection of the marine survey ship "Investigator." Later Kemp (1917, 1925), Nataraj (1942, 1947) and more recently Suseelan (1984) have recorded many more species from the west and east coasts of India and studied their taxonomy. Pelagic shrimp form important food and forage to oceanic tuna, flying fishes (James et al. 1987, Philip 1998) and a number of fish species inhabiting the shelf waters that support commercial fishery. Pelagic shrimp are considered potentially important from the outer shelf and slope waters of the west coast of India (Venkatraman 1960, Suseelan & Nair 1990). Therefore a detailed investigation

was carried out for understanding their distribution pattern, abundance and diel vertical migrations in the eastern Arabian Sea.

### MATERIALS AND METHODS

The pelagic shrimp samples were collected from the DSL during the 10 cruises of FORV *Sagar Sampada* as part of a DSL program in Indian EEZ between May 1998 to December 2000 in the area between 06–21°N and 66–77°E (Table 1). Pelagic shrimp catches were recorded from 123 stations (88%) out of the 140 stations sampled, covering 67 night and 56 day samplings (Fig. 1). The gear used for the collection was the Isaac-Kidd Midwater Trawl (IKMT). The IKMT was designed to collect meso/bathypelagic specimens larger and more active than the specimens caught by plankton nets (Isaacs & Kidd 1951). The towing speed was maintained at 3 knots. Acoustic detection of DSL onboard was facilitated by two Echo sounders (Simrad, EK 400 and EK 200, 38 KHz/120 KHz). The IKMT was operated by observation of the DSL in the echogram, by making an oblique haul for 30 min. The sampling depth varied between 50 and 750 m and hauling depth depended on the concentration of DSL thickness as evidenced from echograms. The catches were preserved in 10% buffered formalin, after noting the volume, immediately after the haul. From the samples pelagic shrimps were sorted out and were used for the study. Identification of pelagic shrimps was done using the

TABLE 1.  
FORV *Sagar Sampada* cruise details.

Cruise #	Months	Year
165	May	1998
167	June-July	"
168	October	"
169	November	"
170	December	"
173	May	1999
174	June	"
183	April	"
185	September	2000
190	December	"

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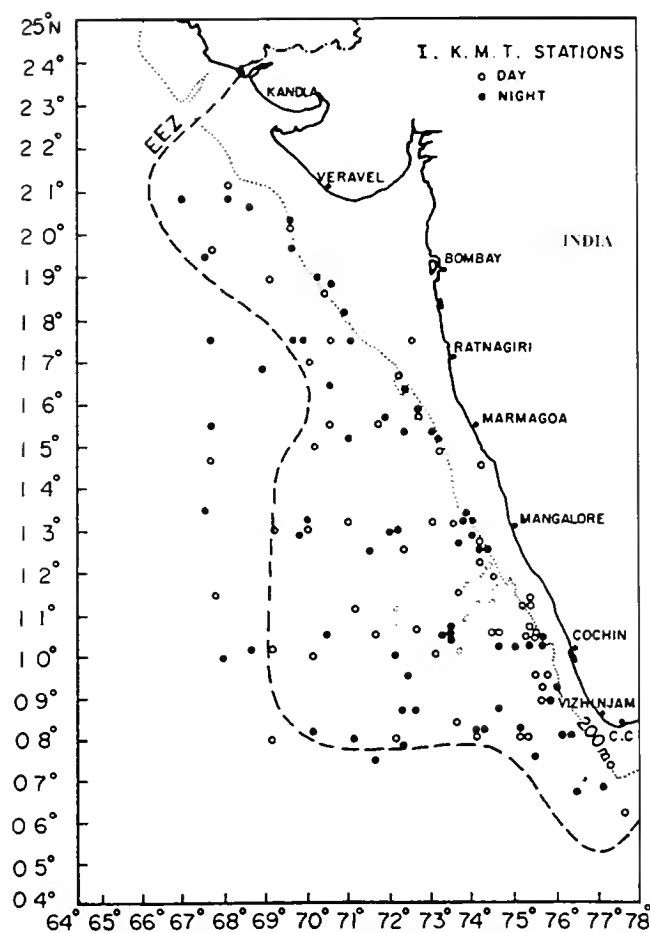


Figure 1. Map showing day and night stations for IKMT collections from the DSL of the Indian EEZ of the Arabian Sea during May 1998 and December 2000.

key and description of species by Alcock (1901), Hansen (1919), Kemp (1920), Holthius (1955), Yaldwyn (1957), Kensley (1971 a, b), Judukins (1978), Burkenroad (1981), Suseelan (1984) and Farfante and Kensley (1997). The pelagic shrimp abundance was estimated using "Swept area" method (Sparre & Vanema 1992). The abundance per 1-degree square was calculated by taking into account the DSL thickness (m), the average numerical abundance of shrimps (No/1,000 m<sup>3</sup>) in the collections taken from that area and these have been extrapolated to compute the biomass for catch

TABLE 2.  
Multilayers in the study of the DSL.

	Depth of the Layer		
	1 Layer (DSL Thickness)	2nd Layer (DSL Thickness)	3rd Layer (DSL Thickness)
10°29'N, 73°30'E	10 m (10 m)	50 m (30 m)	90 (20 m)
08°29'N, 73°32'E	20 m (5 m)	250 m (10 m)	
06°40'N, 77°30'E	40 m (20 m)	90 m (30 m)	400 (100 m)

TABLE 3.

Total DSL biomass (per/1,000<sup>3</sup>) of numerical, volumetric and weight from the DSL biocomposition.

	Day			Night		
	No	Vol	Wt	No	Vol	Wt
Zooplankton	2884	205	297	4990	235	316
Nekton	1049	321	353	1020	252	319
Pelagic shrimp	372	62	63	1480	109	105

1-degree square. Because the sample size was small for a realistic estimation of biomass, the estimation presented here is only an indication of the resource abundance in the surveyed area.

## RESULTS

### DSL Characteristics

The deep scattering layers are found throughout the surveyed area in the Arabian Sea. Single layers are found more frequently than multiple layers. The scattering layer is generally found in depths ranging between 200–750 m during day and surface to 200 m during night, with varying thicknesses and in multiple layers both day and night. The DSL multiple layers were recorded at

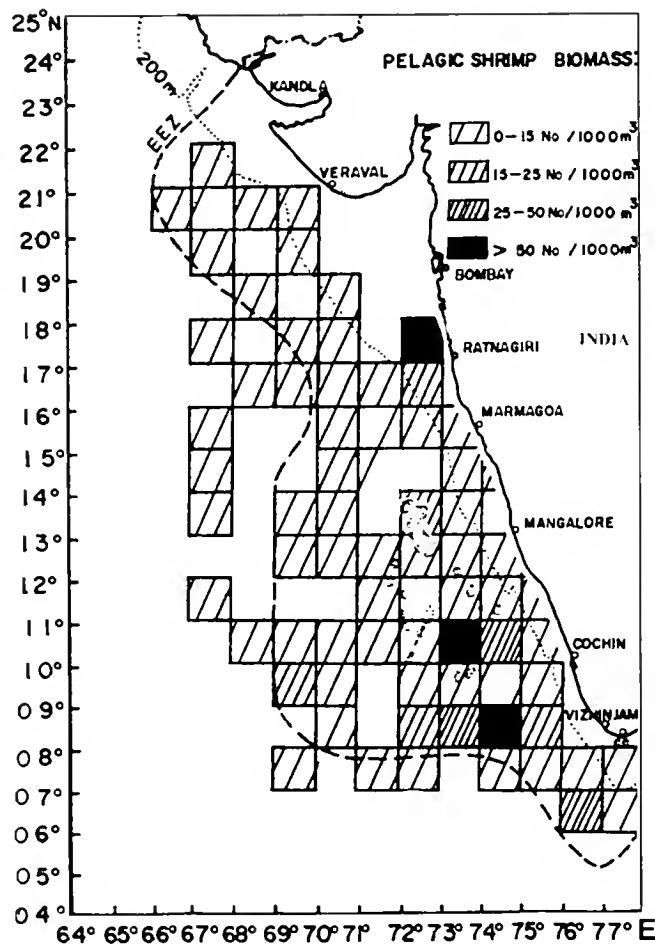


Figure 2. Geographical distribution and abundance (per/1,000 m<sup>3</sup>) of pelagic shrimps



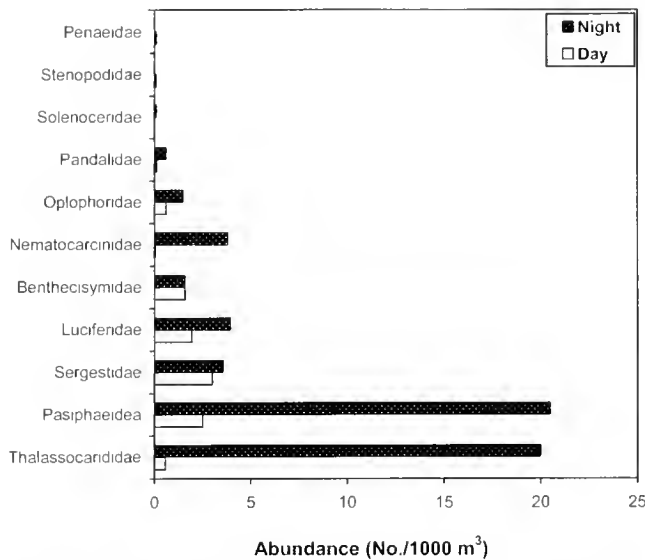


Figure 3. Day and night numerical average abundance of different family of pelagic shrimps

three locations (Table 2) with varying thickness and characters. All three multiple layers were found in the southern regions of the Arabian Sea. The multiple layers invariably recorded in depths between 10–90 m, 20–250 m and 40–400 m. Collections made at different times of day and night indicated that discrete bands or layers ascend to surface from the upper DSL by dusk and during the day it descend down.

#### Geographical Distribution and Abundance of Pelagic Shrimps

There is a distinct resident community of pelagic shrimps found to be widely distributed in the DSL biocomposition. Of the total biomass, pelagic shrimps formed 19% of the total biomass and 47% of the micronekton biomass of the DSL. The result showed that the pelagic shrimps numerically formed one of the major components of the micronekton in the DSL (Table 3). Figure 2 shows the general distribution of the pelagic shrimps (average values for each 1° squares were plotted) in the Arabian Sea. The north (15–22°N)-south (06–15°N) comparison showed an increasing abundance of pelagic shrimps towards south (64.8% of the total catch). The numerical abundance ranged from 0.04–106/1,000 m³. Along the southwest coast the maximum biomass recorded was at 08–74° square (106/1,000 m³) off Vizhinjam and off Cochin (100/1,000 m³), whereas along the northwest coast high catches was recorded off Ratnagiri region (51/1,000 m³). The family-wise estimated average biomass for the whole study area is presented in Figure 3. During the night the families Thalassocarididae and Pasiphaeidea were more abundant (>20/1,000 m³) followed by families Sergestidae (3.94/1,000 m³) and Luciferidae (3.94/1,000 m³), whereas the families such as Oplophoridae, Benthesicymidae, Nematocarcinidae, Pandalidae, Penaeidae, Solenoceridae and Stenopodidae were recorded in small numbers. In the day catches all families were recorded (<2.5/1,000 m³) in small numbers. This showed that pelagic shrimps aggregate mostly during the night.

#### Vertical Migration

Changes in the abundance of pelagic shrimps in the IKMT catches could throw light on the nature of their vertical migration.

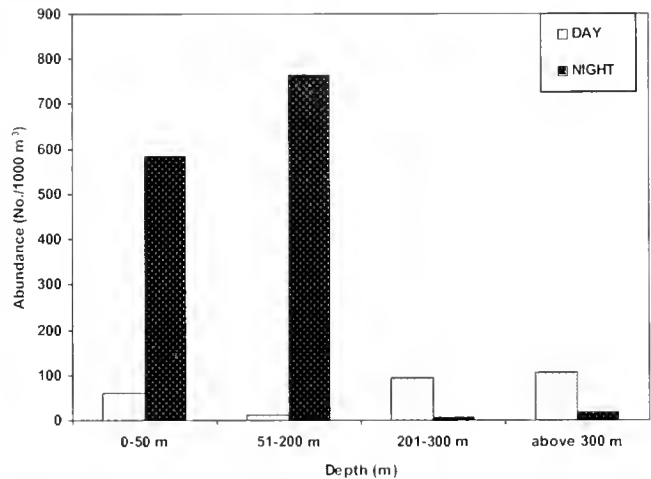


Figure 4. Day and night average abundance of pelagic shrimps (No./1,000 m³)

To study the variation in the vertical distribution of pelagic shrimps, the data arbitrarily were group into 4-depth realm namely, 0–50, 50–200, 200–300 and >300 m for convenience of explanation. The pelagic shrimps abundance was rich at depth-ranges 50–200 m (777/1000 m³) and 0–50 (554/1000 m³) and low catches were yielded from below 300 m. During the night, they were concentrated more in the upper strata (0–200 m) and during the daytime they were more abundant >200 depth (Fig. 4). Among the pelagic shrimps, the dominant groups considered for vertical dis-

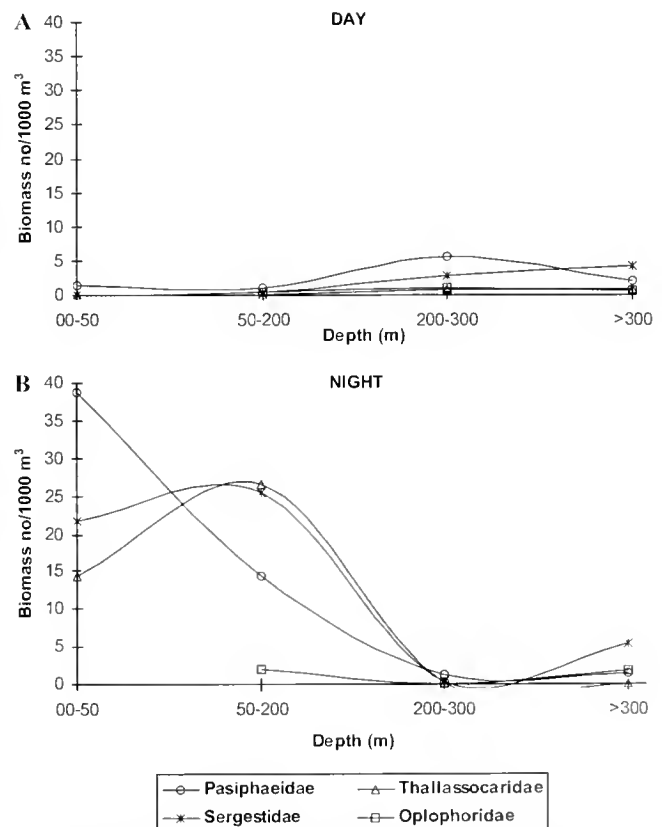


Figure 5. Vertical (numerical average) distribution of important families of pelagic shrimps.

TABLE 4.  
Species composition of Pelagic shrimp and location of occurrence

Species Name	Size Range (mm)	DSL Depth (m)	Latitude (° N)	Longitude (° E)
Intra order: Penaeidea				
Super family: Penaeoidea				
Family: Penaeidae				
1. <i>Pelagopenaeus balboae</i>	60 and 70	40	13° 00'	69° 57'
		50	12° 30'	73° 30'
		200	12° 59'	69° 58'
2. <i>Limulalia danae</i>	60	60	17° 30'	67° 24'
Family: Benthesymidae				
3. <i>Gemmdas praeceus</i>	60	60	07° 07'	77° 12'
4. <i>G. sordidus</i>	20–40	50	07° 07'	68° 32'
		350	10° 31'	77° 12'
5. <i>G. parvus</i>	40	50	07° 07'	77° 12'
6. <i>G. scutatus</i>	26–42	50 to 700		
Family: Solenoceridae				
7. <i>Hymenopenaeus aequalis</i>	25–40	370	13° 09'	73° 40'
8. <i>Solenocera hexa</i>	40	205	16° 30'	72° 14'
Family: Sergestidae				
9. <i>Sergestes semundus</i>	26–46	50 to 350		
10. <i>S. semisus</i>	15–35	50 to 400		
11. <i>S. orientalis</i>	15–35	50 to 350		
12. <i>Sergia mous</i>	30–60	50 to 500		
13. <i>Acetes japonicus</i>	20–25	30	16° 78'	73° 85'
Family: Lucteridae				
14. <i>Lucter typus</i>	0.2–10	50 to 750		
15. <i>L. penicilliger</i>	0.2–10	50 to 750		
16. <i>L. hansen</i>	0.2–10	50 to 750		
17. <i>L. orientalis</i>	0.2–10	50 to 750		
Intra order: Caridea				
Super family: Oplophoridae				
Family: Oplophoridae				
18. <i>Oplophorus typus</i>	6–50	50–750		
19. <i>Acanthephyra sanguinea</i>	30–75	750	11° 26'	67° 34'
		120	10° 31'	68° 32'
		210	09° 59'	72° 01'
20. <i>Meningodora</i> sp.	50	750	14° 31'	67° 32'
		750	11° 26'	67° 34'
21. <i>Notostomus</i> sp.	40–45	750	14° 31'	67° 32'
		750	11° 26'	67° 34'
		100	10° 21'	75° 34'
Super family: Nematocarcinoidea				
Family: Nematocarcinidae				
22. <i>Nematocarcinus tenuirostris</i>	13–24	750	14° 31'	67° 32'
		100	16° 14'	72° 15'
		260	17° 30'	70° 25'
		400	07° 59'	74° 02'
Super family: Pasiphaeidea				
Family: Pasiphaeidae				
23. <i>Leptochela (Leptochela) aculeocaudata</i>	10–30	50–750		
24. <i>L. (Leptochela) robusta</i>	10–35	50–750		
25. <i>Psathyrocaris</i> sp.	35	750	14° 31'	67° 32'
Super family: Pandaloidea				
Family: Pandalidae				
26. <i>Plesionika martia</i>	25–35	100	07° 59'	76° 00'
		180	14° 31'	74° 09'
27. <i>P. alcocki</i>	26–35	180	12° 28'	74° 09'
		280	11° 49'	74° 26'
		245	14° 31'	73° 08'
		350	10° 19'	75° 28'
		400	07° 59'	74° 02'
Family: Thalassocandidae				
28. <i>Thalassocaris armata</i>	5–15	50–750		
Intra order: Stenopodidea				
Family: Stenopodidae				
29. <i>Stenopus</i> sp.	15	30	16° 29'	73° 06'

Species were collected from the area between 06–21° N and 66–77° E.

TABLE 5.  
Swarming species location and their numerical abundance of eastern Arabian Sea

Species	Numbers (/1000/m <sup>3</sup> )	Depths (M)	Latitude	Longitude	Day/Night	Month
<i>Leptochela robusta</i>	25	250	08°29'N	73°30'E	Day	May
	49	10	10°29'N	73°30'E	Night	May
	257	50	10°29'N	73°30'E	Night	May
	99	90	10°29'N	73°30'E	Night	May
	30	100	16°14'N	72°15'E	Night	November
<i>Thalassocaris crinata</i>	28	50	08°30'N	73°25'E	Night	May
	302	75	08°33'N	74°34'E	Night	December
	47	100	10°20'N	73°26'E	Night	December
<i>Sergestes seminuds</i>	29	430	14°29'N	74°09'E	Day	April
	26	40	08°00'N	75°00'E	Day	September

tributional studies were Sergestidae, Pasiphaeidae, Thalassocaridae and Oplophoridae (Fig. 5). Night catches of pelagic shrimps belonging to all families from surface waters (0–200 m) exceeded the day catches. Catches from these depths were higher during the day than at night. Some groups migrate to the shallowest depths possible at night, whereas the deeper-water forms prefer to migrate only to mid depths. For example shrimp belonging to Oplophoridae family were not captured either during day or night from the 0–50 m depth range. They were caught from 200–300 m-depth range during both day and night.

#### Species Composition

The pelagic shrimps of the DSL were made up of 29 species belonging to 19 genera and 11 families. Shrimps belonging to genera *Oplophorus*, *Gennadas*, *Sergestes*, *Leptochela* and *Acanthephyra* were dominant in the DSL catches (Table 4). Out of the 29 species obtained, 16 were restricted to a few locations and 13 were represented along the entire eastern Arabian Sea. The survey results showed that the caridean shrimps, particularly *Acanthephyra sanguinea*, *Meningodora* sp., *Notostomus* sp., *Psathyocaris* sp., *Plesionika martia* and *P. alcocki*, were recorded only from the southern parts. The species *Funchalia dana*, *Solenocera hextii*, *Acetus japonicus*, and *Nematocarcinus tenuirostris* were only from the northern parts and all other species were distributed along the entire Arabian Sea.

#### Swarming Behavior

The swarming nature of pelagic shrimps was evident from the strong concentration on various occasions during this study. In the oceanic waters *Sergestes seminuds*, *Thalassocaris crinata* and *Leptochela robusta* occurred in swarms (Table 5). Swarming nature was noticed mostly in the lower latitudes, and only a few swarms were noticed in the northern region at 16°14'N, 72°15'E. In some of the hauls, shrimps formed more than 75% of the total catch. *Leptochela robusta* was found at 5 locations both during day and night, with maximum abundance at 10°29'N, 73°30'E in the depth of 50 and 90 m (256 & 99/1,000 m<sup>3</sup>) respectively. The *Thalassocaris crinata* was present only in the night collections at 3 locations, and the depth of occurrence ranged between 50–100 m and was caught during May and December with a maximum of 302/1,000 m<sup>3</sup> at 08°33'N, 74°34'E. The species *Sergestes seminuds* was found at two locations during the day collections at depths between 40–430 m during April and September. The abundance was <30/1,000 m<sup>3</sup> for these species.

#### DISCUSSION

The deep scattering layers are found in depth between surface and 750 m with the varying thicknesses and in multiple layers throughout the survey of the eastern Arabian Sea. Barham (1957) found that in Monterey Bay the number of layers changes throughout the year. Herring et al. (1998) recorded distinct multiple layers in the upper 350 m during the daytime off Oman coast. The striking feature noticed during this study was the presence of a high density of pelagic shrimps throughout the eastern Arabian Sea. The quantitative richness of the DSL in the Arabian Sea is mainly caused by the presence of micronektonic organisms like pelagic shrimps, swarming crabs, cephalopods and epi-mesopelagic fishes. Among the pelagic crustaceans occurring in the scattering layers of the sea, shrimps appear to occupy a prime position numerically (Suseelan & Nair 1990). Their distribution in fact shows a clear north-south variation as the abundance increased from north to south. The southern region was found to be more productive compared with the northern region of Arabian Sea, where their abundance was rather patchy and productive areas were less extensive. Overall biomass estimations showed that the maximum population densities were recorded off Vizhinjam (106/1,000 m<sup>3</sup>) and off Cochin (100/1,000 m<sup>3</sup>). Of the total 19% of the pelagic shrimps recorded from the DSL, species such as *Leptochela robusta*, *Sergestes seminuds* and *Thalassocaris crinata*, which are of high forage value, appeared in seasonal swarms during day/night. Their swarming is strongly seasonal and the fishing seasons correspond with the swarming periods. A study on the day-night variations in abundance revealed a pronounced vertical migration of these animals, being more concentrated in night samples than day. Herring et al. (1998) found that decapods (particularly species of *Gennadas*, *Plesionika* and *Sergestes*) contributed more to the biovolume among the micronekton of the DSL. Shrimps feed actively at night, however physiological evidence suggests that the lower meso and bathypelagic species are able to maintain their predatory activities throughout range of migration, by day as well as by night (Pearcy & Forrester 1966). Waterman et al. (1939) stated that diurnal vertical migrations have long been known to play an important part in the lives of pelagic organisms. At night the animals were more densely packed in the depth ranges of 0–200 m, whereas, during the day these animals usually appeared at depths >200 m. Diel vertical migrations of pelagic shrimps are often extensive, hence accelerating the vertical flux of organic matter and recycling of chemical substances through the water column (Kikuchi & Omori 1985). Because fish such as tuna feed on pelagic shrimps, a positive

relationship between the abundance of these two groups could be expected (Alverson 1961). Among caridean shrimps recorded during the present study, a species that deserves special attention is *Leptochela robusta*, which was caught in large quantities throughout the west coast. This species is reported to form important forage to tuna and other pelagic fishes in the Lakshadweep and neighboring seas (George & Paulinose 1973, James et al. 1987). According to George et al. (1977) the maximum density of tuna and allied fishes in Indian EEZ is in the southwest coast and the oceanic islands. It is therefore reasonable to presume that the high productivity of pelagic shrimps might influence the abundance of tunas and allied fishes in these regions. The observation was made by James et al. (1987) that oceanic tunas that feed heavily on

*Leptochela robusta* is indicative of selective feeding of tunas on such species of pelagic shrimps. Voracious feeding on species of these genera by tuna (albacore) and fin and sei whales (*Balaenoptera borealis*) has been reported from the Pacific waters (Omori & Kawamura 1972, Omori 1974).

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## INDICATORS OF PERFORMANCE IN THE FISHERY FOR SHRIMP *PALAEMON SERRATUS* (PENNANT) IN IRISH COASTAL WATERS

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**ABSTRACT** A commercial fishery for shrimp *Palaemon serratus* occurs on the south and west coasts of Ireland. Two temporal phases were identified: expansion from the mid 1970s to the late 1980s when maximum national landings were less than 150 t annually; and a second phase since 1990 when most years yielded more than 200 t. Maximum landings of 548 tons were reached in 1999. Three indicators of fishery performance are examined: total annual landings, weight landed per vessel per day and the average weights of individual shrimp in October and December, reconstructed from processors' records of graded shrimp. Total landings and daily landings weights correlated positively, whereas both correlated negatively with average individual shrimp weight in October and December over a 13-year period. Periodic and possibly cyclical recruitment events are identified as a contributor to improved yield.

**KEY WORDS:** *Palaemon serratus*, *Palaemon elegans*, Ireland, inshore, shrimp fishery

### INTRODUCTION

A trap (creel) fishery for shrimp commenced in the vicinity of Baltimore, southwest Ireland in the mid 1970s (McPadden 1979). It subsequently expanded eastwards along the south and northwards along the west coasts. The species making up the majority of the landings was *Palaemon serratus* (Pennant), the smaller *P. elegans* accounting for less than 1% of landings by number (Fahy et al. 1998a). Cole (1958) and Forster (1951) described *P. serratus* as having a pronounced southern distribution in Britain and the majority of landings in Ireland came from the south west (Fahy & Gleeson 1996) (Fig. 1).

Shrimp are fished in plastic Chinese-hat-ended traps baited with whitefish or pelagic fish offal. Berried females fetch the best prices and are targeted by the fishery. These are most accessible and concentrated inshore during the autumn when growth is fast and an individual could double its weight in four to five months (Fahy & Gleeson 1996). However, shrimp are poorly selected by the square mesh used in creels (Fahy et al. 1998b); all sizes above 55 mm total length (which was the threshold at which recruitment commenced) are generally retained.

The growth and longevity of *P. serratus* have been investigated by a number of workers: Sollaud (1916) believed the species lived for five or six years; Cole (1958) thought it probable it survived for four but Forster (1951, 1959) favored two and Figueras (1986) described the expected life span as three years.

Significant elements in the life cycle reconstructed from data in Fahy and Gleeson (1996) and corroborated in Fahy et al. 1998b are presented in Figure 2 on which the traditional fishing season (August to January) is marked with rectangles whose shading indicates its relative importance to the industry (0-group shrimp are harvested and sold but they realize low prices, shrimp in their second autumn are the principal target and are most valuable, whereas female shrimp that might be in their third autumn are infrequent). Shrimp hatch in May and first recruit to the creels the following August and September. Discarding was an exceptional practice during the period under review (the majority of fishers sold the unsorted contents of their creels), hence 0-group animals were retained in the landings. Currently fishing also takes place up to 6

n m offshore when the females migrate into deeper water during the colder months after the end of the year.

### MATERIALS AND METHODS

Total landings of shrimp were collected by the government department responsible for fisheries (currently the Department of Communications, Marine and Natural Resources) on a national scale (Fig. 3).

Details of shrimp purchases and grading were obtained from five processors, labeled A to E. These processors purchased shrimp from a wide geographic range and several contemporaneously bought landings from the same area. Processors mechanically sorted shrimp using vibrating screens into four or five size grades at least five 1-kg samples of each of which were obtained and analyzed in the Marine Institute.

In the laboratory samples were disaggregated by sex; females have a faster growth rate than males. Individual measurements of total length from the tip of the rostrum to the end of the telson were noted (0.1 mm), the shrimp having been straightened against a rigid surface and individual weights (0.1 g) were recorded. Length frequencies separated by sex were predominantly bimodal with the two modes interpreted as the 0 and 1-y groups. However some females exceeding 24 mo in age may also have been present but not identifiable in the length frequencies. The bimodal length frequencies were sliced (separated at the lowest length frequency) to divide year classes. The consequence for the average shrimp weights of altering the proportions of the two age groups was modeled.

Definitions of grades varied from one buyer to another and the average weights of corresponding grades among processors differed; to maintain consistency of product, a processor maintained his sorting screen characteristics from one year to another. Table 1 contains the average weights of shrimp per grade analyzed by the Marine Institute and used throughout the period of the investigation to estimate the average individual weight of shrimp landed.

Processors provided details of the weight composition of graded shrimp in the landings they purchased (the actual weights of grades 1–4 or 5 after screening) together with dates and places of purchase, and from these and the average weight of shrimp within a grade the average weights of shrimp captured from an area in a specific time period was calculated according to the formula:

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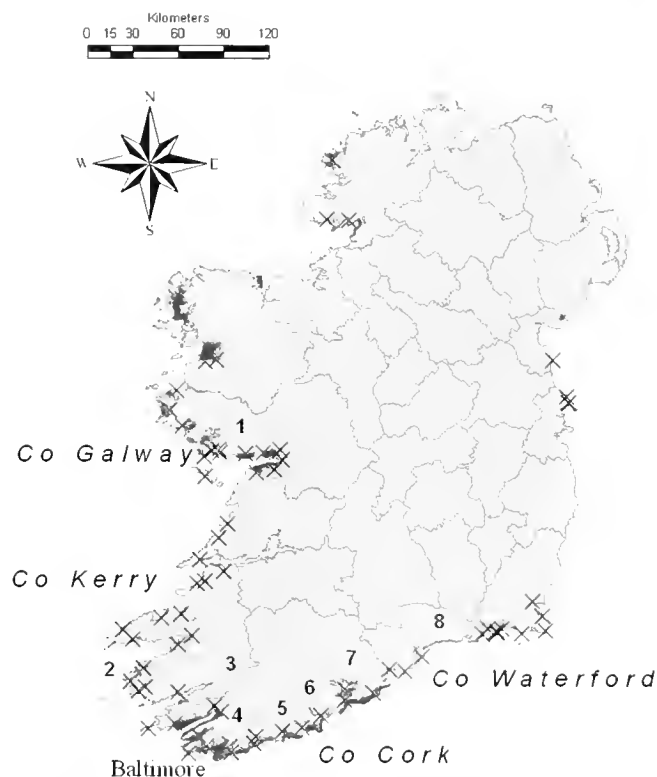


Figure 1. Map of Ireland on which the county boundaries are marked, showing the extent of inshore shrimp fisheries (black) and places where shrimp landings were recorded by the Department of Communications, Marine and Natural Resources (DCMNR) between 1995 and 2003 inclusive (x). Numbers 1–8 refer to the areas from which data on shrimp grading and daily landing weight per vessel were obtained. Places referred to in the text are identified.

$$\sum_{i=1}^n (GrWt_i * AvWt_i)$$

where  $GrWt_i$  is the proportion of stock at this grade and  $AvWt_i$  is its average individual weight,  $i$  was the largest and  $n$  the smallest grade.

The time period to which average weights referred was dependent on factory procedure: landings might be sorted daily or weekly and some archived data were for landings bought-in over a month.

The average individual weight in October and December was calculated using all consignments in these months. Grading was not always undertaken by a processor and such data as existed were not always made available. In 1991 grading details of 3% of total landings were obtained; the following year this increased to 98%. Thereafter it fluctuated between 20% (in 1993 and 2001) and 50% (in 1995), averaging at 35% over the period. In all 162 estimates of October and December weights were made and these were distributed among 92 combinations of processor, area and year from 1991 to 2003 (Table 2).

The majority of shrimp were fished mainly by small inshore vessels (<10 m) crewed by two persons fishing 300–500 creels. Logbooks were not maintained in this fishery. Records of daily landings by a single vessel, which were then consigned to a processor, were obtained from the same geographical range and from the same companies by scrutiny of payment records; individual boats were not identified. An estimated 3% of all landings were

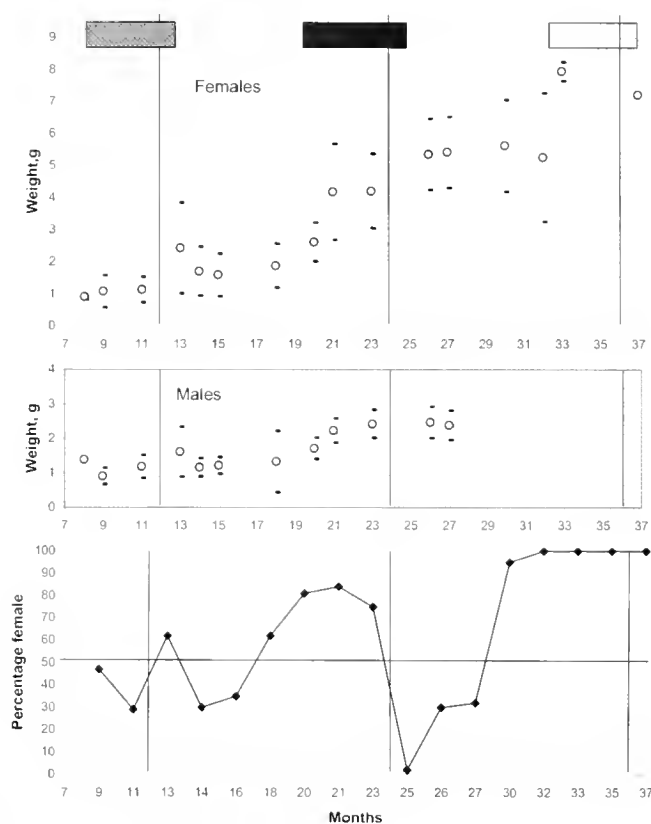


Figure 2. Elements of the shrimp life cycle of relevance to the inshore autumn commercial fishery whose duration (August to January) is represented by rectangle on the top panel. Relative importance of successive years is indicated by intensity of shading. Growth of females and males is shown as the mean  $\pm$  1 SD of the disaggregated and sliced length frequencies converted to weight. The last panel shows the sex ratio (from Fahy & Gleeson 1996 and Fahy et al. 1998b).

reported as daily landings in 1991 and 25% the following year. An average of 13% of total landings were reported as daily landings overall. Daily consignment numbers averaged 1,076 annually and ranged from 347 in 1991 to 1,906 in 1994.

The areas from which shrimp were purchased (numbered 1–8) are shown in Figure 1. Area 1 is in County Galway, area 2 is County Kerry, areas 3–7 are in County Cork, and area 8 is County Waterford.

The statistical package used in the work was SPSS 11.0.

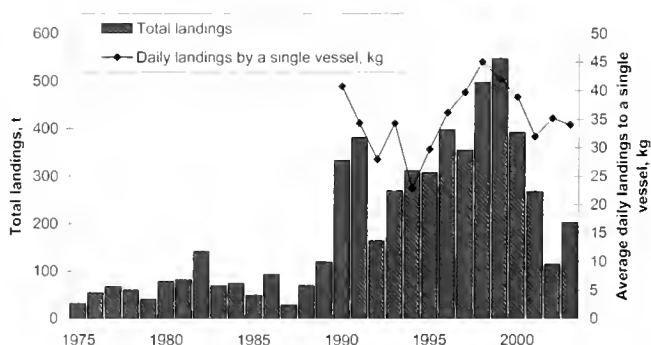


Figure 3. Total landings (Source: DCMNR), 1975 to 2003 and annual average weight of daily landings by vessels fishing shrimp, 1990 to 2003.



TABLE 1.

Average individual weights (g) of shrimp in grades sorted by five processors. The averages are of five or more samples of each grade.

Grades/Processors	A	B	C	D	E
1	7.4	9.3	6.2	8.3	11.5
2	4.7	7.0	4.5	5.9	7.2
3	3.3	2.4	3.0	3.7	4.4
4	2.2	1.8	1.5	2.9	2.0
5				2.2	

## RESULTS

The *Palaemon* shrimp fishery in Ireland is conducted close inshore in the autumn and it is concentrated on the south west of the country. Few shrimp are harvested in the Irish Sea (Fig. 1). A fishery exists in the northwest where shrimp accumulated in storage boxes are periodically collected for processing, which is also carried out mainly in the southwest. Between 1995 and 2003 the counties that provided the data on which this investigation is based recorded 93% of the total landings of shrimp (Galway, 20%, Kerry, 23%, Cork 45% and Waterford, 6%).

The fishery targeted 1-group shrimp (Fig. 2), especially females, which are twice as heavy as males during their second autumn. The fishery was mainly conducted from August to January when 1-group females moved close inshore. Females outnumber males in landings, the majority of which are harvested within 1 n m of the coast. Not all females have reached their asymptotic length at this age however and they continue their growth in deeper water during the colder months. Very few females survive to contribute to the next fishing season. Investigations on shrimp in southwest Ireland in the mid1990s supported Forster's interpretation of life expectancy for practical assessment purposes although the longevity of some few females might exceed 24 mo (Fahy & Gleeson 1996).

Annual landings (Fig. 3) had 2 phases: a period of low catches up to 1989 (142 t in 1982 was exceptional) and a subsequent period of expanded catches; other than in 1992 when they totaled 164 and in 1993 269 t, landings of shrimp exceeded 300 t per year during the latter period. In 1999 landings peaked at 548 t after which they fell to 115 t in 2002, recovering again to 413 t in 2004.

Mean daily landings per boat over the period were 34 kg (ranging from 23 kg in 1992 to 45 kg in 1998) and analysis of weight frequency over the period recorded 90% was less than 75 kg ( $n =$

12,669, between 1990 and 2003). Landings of as low as 2 kg were recorded and the upper end of the range was at 250 kg (Fig. 3). Daily average consignment weights correlated significantly with total landings ( $r^2 = 0.353$ ,  $n = 14$ ,  $P = 0.0251$ ).

All processor/area/year combinations of data covering the period September to December were considered in this analysis and for these the average individual shrimp weights in October and December were calculated. Selections of these data sets are shown in Figure 4 to illustrate the nature of the data. In the majority of panels there was some increase in weight in the autumn months and the average individual weight frequently declined coinciding with the move of females into deeper water in the colder months.

Forster (1951), Cole (1958), Sollaud (1916) and Desbrosses (1951) identified a number of variations in growth and biology of *Palaemon* shrimp, which might be attributed to location (sex ratio favors females in rocky areas and males where the substratum provides less cover, for example) so the average individual weights were examined for bias, which might be linked to processor or area. Average individual weights were higher in areas 1 (4.95 g) and 8 (4.92 g) than elsewhere (3.9–4.2 g). In the rocky environment of Connemara (area 1) shrimp are known to be of better commercial quality because of the higher incidence of berried females. All records from area 1 were provided by processor D. Two processors, A and B, handled shrimp from area 8 and both provided a similar weight range. Area 8 includes the only area in which fishers were known to have graded their catches. An analysis of variance found a significant difference between processor and average shrimp weight ( $df = 3$ ,  $F = 3.420$ ,  $P = 0.019$ ) and between area and average shrimp weight ( $df = 6$ ,  $F = 2.740$ ,  $P = 0.015$ ) but average shrimp weights in samples from the same area handled by different processors did not differ significantly ( $df = 4$ ,  $F = 1.685$ ,  $P = 0.157$ ).

Areas 1 and 8 had higher average shrimp weights, but data from these areas were more plentiful in some years than others hence, when they did occur they might have distorted the results in those years, so samples from areas 1 and 8 were excluded from all further analyses, and 147 average individual shrimp weights were used in the remaining calculations.

Average individual shrimp weights displayed periodic trends during the study period: between 1991 and 1995 average individual weights in October and December rose ( $x\text{-var} = 0.365$ ;  $n = 29$ ,  $r^2 = 0.0384$ ,  $P = 0.0003$ ), from 1995 to 1998 they declined ( $x\text{-var} = -0.189$ ,  $n = 30$ ,  $r^2 = 0.152$ ,  $P = 0.033$ ) and from 1998 until 2001 they increased again ( $x\text{-var} = 0.315$ ,  $n = 52$ ,  $r^2 = 0.270$ ,  $P < 0.0001$ ) (Fig. 5). A trough occurred in 1998 and the average weights tended downward again, though not significantly, between 2001 and 2003.

Average individual shrimp weight correlated negatively with daily consignment weight ( $n = 147$ ,  $r^2 = 0.0816$ ,  $P = 0.0005$ ) and national landings ( $n = 147$ ,  $r^2 = 0.0711$ ,  $P = 0.001$ ).

A correlation of average shrimp weight with time, superimposed on the data in Figure 6, suggests an increase in average shrimp weight over the period of the study.

The relationship between the proportion 0-group and average individual shrimp weight was modeled on the composition of shrimp samples provided in October and November by processor A. Disaggregated into males and females the length frequency compositions were sliced into 0 and 1-groups. In the model the proportion of 0-y shrimp was increased and the consequent aver-

TABLE 2.

The origin of average individual shrimp weights in October and December.

Processors/Areas	1	2	3	4	5	6	7	8	Totals
A		7	2	9	5	4	2	7	36
B							7	9	16
C						2	2		4
D	11								11
E		13	8		4				25
E		13	8		4				25
Totals	11	20	10	9	9	6	11	16	92

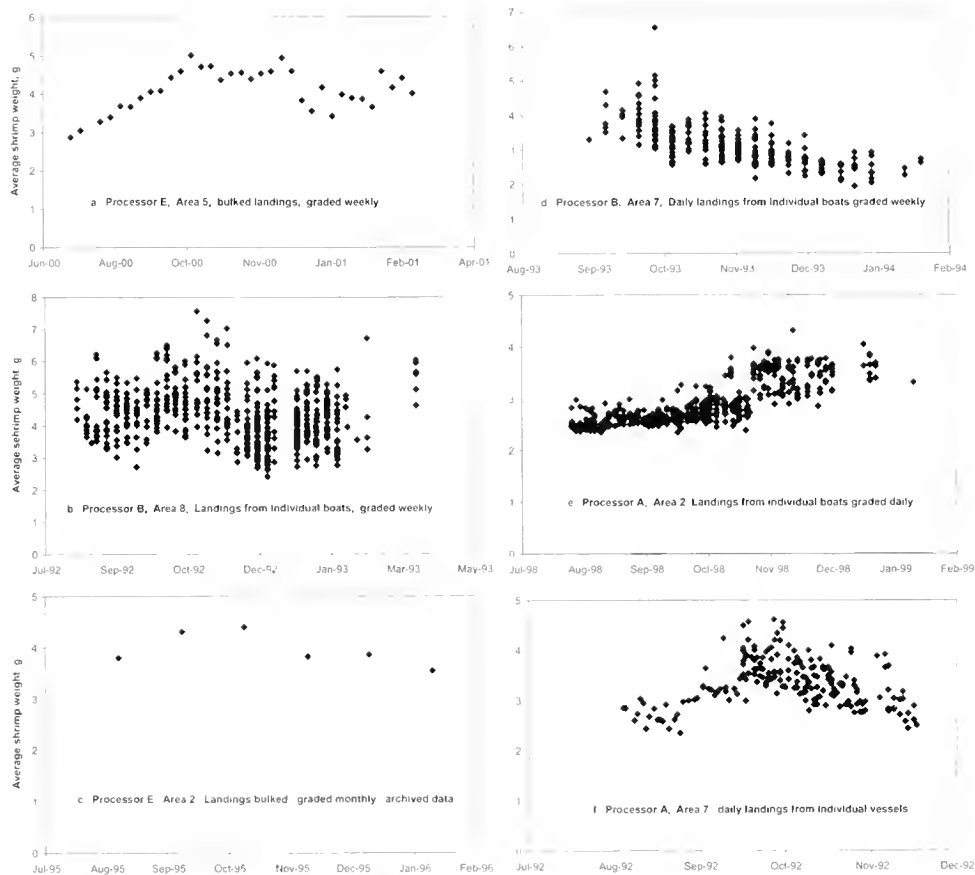


Figure 4. Examples of the seasonal distribution of average shrimp weights in landings from four areas in five years. On each the area and processor are identified. The examples are selected to illustrate the type of data used in the calculations: panel a, is of bulked daily landings, which were graded on a weekly basis, b and d are individual daily landings, which were graded on a weekly basis, e and f are daily landings, which were graded on a daily basis and c is graded material archived on a monthly basis.

age individual shrimp weight was noted (Fig. 7). An inverse relationship between average shrimp weight and the proportion of 0-group shrimp also suggested that the recruitment of 0-group shrimp influenced yield.

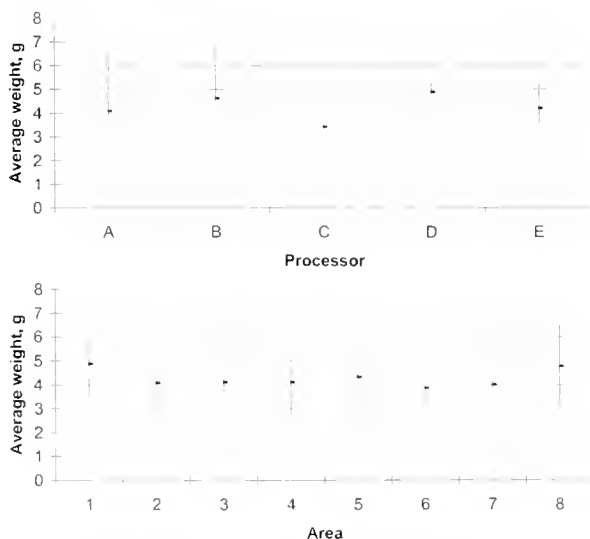


Figure 5. Range and mean values of average shrimp weights in October and December as reported by five processors (above) from eight areas (below).

## DISCUSSION

Expanding from the mid 1970s, the inshore fishery for shrimp reached its maximum geographical extent in the early 1990s. The fishery is unregulated.

The maximum average individual weight in a shrimp catch from the inshore fishery is recorded in the late autumn, between October and December. Average individual weight is influenced by the sex ratio within the catch, whether it has been sorted to remove 0-group individuals and the relative proportions of 0 and 1-group animals represented (Fahy & Gleeson 1996, Fahy et al. 1998b).

Annual shrimp landings correlated positively with daily landings suggesting that the latter reflected changes in shrimp abundance. Greater abundance of a target species attracts additional fishing effort and that, in turn, increases yield. While logbooks were not maintained in this fishery, it is possible to obtain an estimate of fishing effort from the average daily and total annual landings. Using these data fishing days annually would have averaged 9,266 between 1991 and 2003 and ranged from 3,263 in 2002 to 13,650 in 1994. Fishing effort is strongly correlated with total landings ( $n = 13$ ,  $r^2 = 0.6919$ ,  $P = 0.0004$ ).

Successful fishing could be based on a strong 1-y class of higher first sale value or an abundant 0-y class contributing greater volume but less unit value. In the analysis annual landings and daily consignment size both correlated negatively with average shrimp weight in October and December suggesting that increased

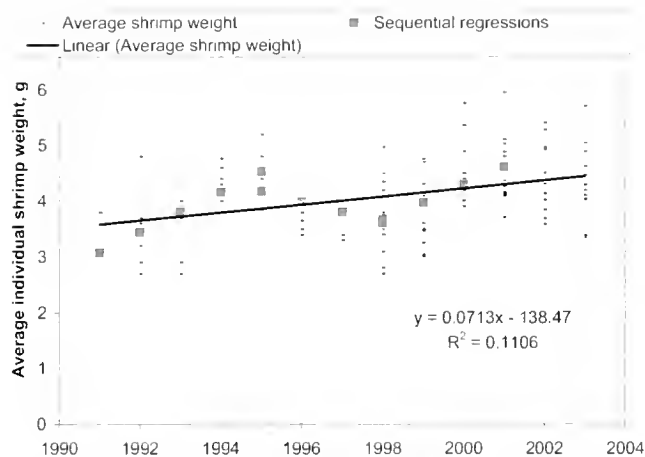


Figure 6. Trends in the average weights of shrimp in October and December, 1991 to 2003. Short-term sequential regressions and the long-term linear trends are shown and the variables for the latter are given.

landings were caused by greater proportions of 0-group shrimp and increased recruitment.

Figure 7 illustrates how a decreasing average shrimp weight signals this recruitment but is not a sensitive indicator. Modeled average shrimp weights were 5.45 g when they consisted of only 1-group animals; demonstrable change was brought about by the addition of an equal weight of 0-group shrimp, which reduced the average weight to approximately half (2.85 g). Doubling the addition of 0-group shrimp further reduced the average weight to 2.42 g (by a further 0.44 g) and tripling it reduced the average weight to 2.27 (by an additional 0.15 g). In terms of numbers, working from the data shown in Figure 2, the addition of 6:1 0-group to 1-group could increase the total weight of a consignment in the autumn fishery by 300%.

An investigation of the commercial shrimp fishery (Fahy & Gleeson 1996) provided some analyses of the age and sex composition of graded shrimp but had limited access to quantitative factory grading data. Without disaggregating for sex, they sliced the length frequency distributions at three points (65, 70 and 75 mm) to separate the 0 and 1-groups. At the time (early 1990s) there would have been a low incidence of 0-group shrimp in the landings anyway, but the slice point was also too far to the left, 85 mm would have been more appropriate. Fahy and Gleeson's review of the fishery in the 1990s considered it well managed. However, they noted the tendency for the autumn fishery to extend over a longer period, a trend that has since accentuated. The pursuit of shrimp offshore during the colder months of the year has also intensified as stock depletion of other inshore species and the capitalization of the inshore sector have progressed.

Fahy and Gleeson (1996) reported that shrimp are berried for much of the year: 1-group females become ovigerous in the autumn but 0-group come into berry the following spring. Berried

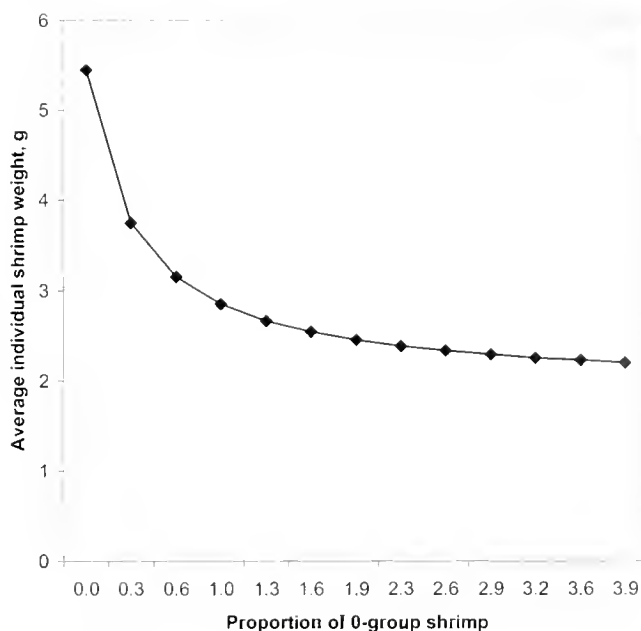


Figure 7. Modeled inverse relationship between average shrimp weight and the proportion of 0-group shrimp in the landings.

females are the most commercially valuable category of shrimp and confining the fishery to the autumn allowed the surviving 0-group cohort to reproduce. They expressed concern that a fishery extending over much of the year could deplete shrimp spawning stock biomass.

The gradual upward trend in average weights since the fishery completed its full expansion in the early 1990s (Fig. 6) may indicate a trend towards lower recruitments. Episodic larger recruitments are associated with shrimp of small average size and low value; larger shrimp are more valuable and their exploitation offshore when stocks are at a low level combined with their exploitation during the spring reproductive phase could culminate in growth overfishing, and this may now be a reality. A precautionary approach to prevent growth overfishing is required and an obvious initiative would be the restoration of the traditional fishery confining exploitation to inshore waters in the autumn months.

#### ACKNOWLEDGMENTS

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## DISTRIBUTION OF THE SEA URCHIN *HEMICENTROTUS PULCHERRIMUS* ALONG A SHALLOW BATHYMETRIC GRADIENT IN ONAGAWA BAY IN NORTHERN HONSHU, JAPAN

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**ABSTRACT** Distribution of the sea urchin *Hemicentrotus pulcherrimus* was monitored monthly or bimonthly from April 1998 to May 1999 at a 100 m<sup>2</sup> permanent site in intertidal to subtidal zones at depths of up to 2.8 m in Onagawa Bay, northern Honshu, Japan. Factors causing seasonal demographic changes were analyzed from vertical algal vegetation, sea urchin size, gonad development, and gut content. The highest density of *H. pulcherrimus* was found on algal turf on boulders at the depth of 0–0.3 m. Migration of adult sea urchins on crustose corallines at the depth of 0.3–2.8 m to the algal turf and subsequent inhabitation in more suitable boulder habitat are suggested from November to March, when *Acrosorium polyneurum*, which is their main food, grew dominantly. As the high gut content of crustose coralline of the sea urchins on crustose corallines resulted in significantly lower gonad indices, it is likely that migration is caused by their high foraging activity for *A. polyneurum* to ensure gonad development. The growth of the sea urchins on algal turf was ensured by *Chondrus* spp. as main food.

**KEY WORDS:** sea urchin, *Hemicentrotus pulcherrimus*, distribution, gonad development, gut content, migration, *Acrosorium polyneurum*.

### INTRODUCTION

The sea urchin *Hemicentrotus pulcherrimus* (A. Agassiz 1863) is found in intertidal and subtidal zones from Kyushu to Rebun Island, Hokkaido, Japan and in Korea and China (Shigei 1995, Agatsuma 2001a). This sea urchin is an important fisheries resource in shallow waters in the coastal regions of the southern Japan Sea from Fukui Prefecture. The brands “Echizen Uni” in Fukui Prefecture and “Shimonoseki Uni” in Yamaguchi Prefecture are well known. In Fukui, stones were introduced in 1910 to enhance the sea urchin’s habitat (Andrew et al. 2002, Agatsuma et al. 2003). Adult sea urchins with poor gonad development have been transplanted to kelp forests, where gonad development improves (Andrew et al. 2002, Agatsuma et al. 2003). In addition, seed production was initiated in a Yamaguchi hatchery in the 1960s (Sakai et al. 2003). The seeds (juveniles) of *H. pulcherrimus* were released in Saga, Shimane and Fukui Prefectures, and the number exceeded 1000,000 individuals in 1993, releases ranged from 300,000–890,000 individuals until 2000 (Agatsuma et al., 2003).

Quantifying seasonal patterns in density and size distributions among different habitats is necessary for managing and sustaining populations of sea urchins. In addition, the seasonal habitat selection relative to age and associated with algal assemblage as food resources that ensure growth and gonad development should also be clarified. In shallow waters in the Sea of Japan, Hokkaido, 0-y-old *Strongylocentrotus nudus* inhabit crustose coralline communities where the larvae settle (Taniguchi et al. 1994, Sano et al. 1998) throughout the year. Sea urchins that are >1 y old migrate to the kelp *Laminaria religiosa* bed in an intertidal zone, where their gonads are ensured to develop for reproduction from summer to autumn. They then return to the crustose corallines to avoid the strong wave action in winter (Agatsuma 2001b). A similar seasonal migration of *S. nudus* is also found in Pacific coastal waters, where an *Eisenia bicyclis* forest grows in subtidal shallow waters (Sano et al. 1998, Sano et al. 2001). Adult *Paracentrotus lividus* migrate from a pebble area where larvae settle to a *Cymodocea*

*nodosa* sea grass bed in relation to a change in food habit (Fernandez et al. 2001). The migration of *Sphaerechinus granularis* from *Phymatolithon* communities to *Zostera marina* or macroalgal beds is also related to changes in food habit (Guillou & Michel 1993). *Loxechinus albus* with >45 mm diameter also migrate from the intertidal zone where larvae settle to brown macroalgal beds at a depth of 4–15 m (Contreras & Castilla 1987). However, except for *S. nudus*, the principal purpose for migration is not clear. And that, there is no known reason for sea urchins to migrate, and the foods that they forage have not been identified by analysis of their gut content. In the life history of *H. pulcherrimus*, seasonal changes in the vertical distributions and the algal foods in relation to algal vegetation are also unknown.

In the present study, we monitored the density and test diameter of a *H. pulcherrimus* population in relation to the depth, bottom characteristics, and vertical algal vegetation from intertidal and subtidal zones in the Pacific near shore waters of northeastern Honshu. An analysis of the gut content was undertaken to determine the foods for gonad development and growth. Consequently, the factors contributing to seasonal demographic changes were suggested.

### MATERIALS AND METHODS

#### Site Characteristics

A monthly or bimonthly survey was conducted in the intertidal and subtidal zones at Sashigahama (38°28'N, 141°29'E) in Onagawa Bay, Miyagi, from April 1998 to May 1999 (Fig. 1). Previously, a study site was determined from predominance of *H. pulcherrimus* by SCUBA diving observation. A survey was performed using one fixed transect (50 m long × 2 m wide) as the study site placed perpendicularly to the coast line.

In April 1998, the bottom profile at the study site was sketched precisely using a 1 × 1 m quadrat by SCUBA. Depth was measured with a water-pressure gauge (Japan Aqualung Co., Ltd.) at an interval of 1 m along the leaded rope placed at the center of the study site, and compensated at the level of the shallowest water. Algal vegetation at the study site in each quadrat was observed

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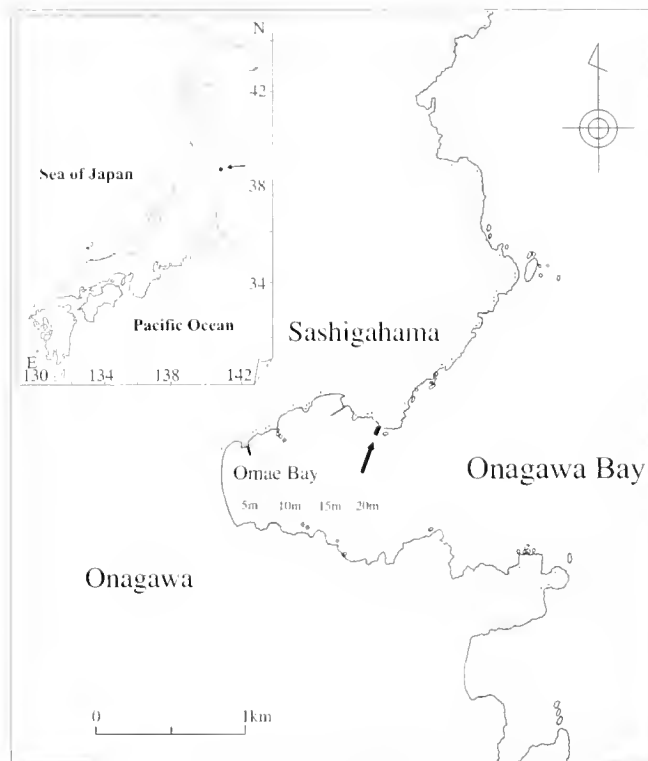


Figure 1. Location of the study site in Onagawa Bay in northern Honshu, Japan.

using SCUBA and video-recorded (Handycam, DCR-TRV7, Sony Corporation). Unidentified algae in the field were collected and identified in the laboratory.

The mean surface water temperature every ten days during the present study and the averages for 30 y from 1968–1997 were referred to the data at Enoshima (38°24'N, 141°36'E) near the study site, measured by Miyagi Prefecture Fisheries Research and Development Center.

#### Density and Size Analysis

In the study site, a 1 × 1 m quadrat was used, and the test diameter of *H. pulcherrimus* was measured in each quadrat with a vernier caliper (1 mm accuracy); the results were recorded on aqua-notes by SCUBA. The sea urchins were replaced in the same quadrates. No survey was performed at a distance of 0–25 m in January 1999 because of the high wave action.

*Strongylocentrotus nudus* and *S. intermedius* also distributed in the study site and their density in each quadrat was also recorded. Throughout the year, *S. nudus* was found on deep crustose corallines with density of 4.5–8.4 individuals/m<sup>2</sup>. Low density of <2.5 individuals/m<sup>2</sup> was found for *S. intermedius*.

#### Gonad Analysis

To determine the gonad development and feeding habits during the study, except for April 1998, a 50 m leaded rope was set up at a distance of 15 m west of the site, where similar algal vegetation was observed. Twenty specimens of *H. pulcherrimus* larger than 3 cm in diameter, which exceeds the mature size (Kawana 1938), were collected from algal turf along the line. Similarly, 5–10 specimens were collected at crustose corallines. The test diameter and body weight (g wet weight) were measured using a vernier caliper

(0.1 mm accuracy) and an electronic balance (0.01 g accuracy). Gonad wet weight was also measured (0.01 g accuracy), and gonad index (gonad weight × 100/body weight) was calculated. Part of the gonad was preserved in 20% formalin for 24 h, and after that, in 10% formalin. Using standard histological techniques, serial cross sections (6 µm) were cut through the center of a gonad and stained with Mayer's hematoxylin and eosin. Histological sections were classified according to Fuji (1960): Stage I, recovering; Stage II, growing; Stage III, premature; Stage IV, mature; and Stage V, spent.

#### Gut Content Analysis

Five specimens with a test diameter in excess of 3 cm were used to analyze the gut content on algal turf and crustose corallines. The gut content for each individual was obtained and filtered on a nylon net with a mesh size of 350 µm. The component of fractions <350 µm was excluded because it was difficult to sort. First, the components were divided into three groups, algae, animals and minerals, according to shape, color, and hardness with the use of a stereomicroscope. Furthermore, algae were classified into a family, genus, or species from its specific cell structure using a dissecting microscope. After that, each food item sorted was sucked on filter paper (Whatman GF/C) and dried for 12 h at 80°C in a hot-air convection oven and then weighed using a microbalance (1 mg accuracy).

#### Statistical Analysis

Statistical difference in densities of *H. pulcherrimus* at each four of the characterized areas in the study site among months and the densities among four areas was analyzed by two-way repeated ANOVA and Scheffé's multiple comparison test. Significant difference in the gonad indices on algal turf and crustose corallines among months was analyzed by one-way factorial ANOVA and Scheffé's multiple comparison test. The difference in indices between the two vegetation types were analyzed by unpaired *t*-test after confirming no significant difference in variance between them by *F*-test. All the data showed normal distribution and homogeneous variance by Shapiro-Wilk's *W*-test and Cochran's test, respectively.

## RESULTS

#### Site Characteristics and Seawater Temperature

At the study site, boulders formed >50% of the substrata at the study site at distances of 10–17, 25–34, and 42–50 m. In the other areas, undulating rocks with ditches and holes in addition to boulders were present (Fig. 2).

The depth and seasonal changes in the vegetation of the dominant algae with these coverage of ca. >50% in the study site are shown in Figure 3. The area at a distance of 0–10 m is in the intertidal zone. Subsequently, the area sloped gently from 10–24 m at depths of up to 0.3 m and steeply from 24–50 m at depths of 0.3–2.8 m. From the upper to the lower intertidal zone, *Gloiopeltis furcata*, *Anelipes japonicus*, and *Sargassum fusiformis* grew all year round. *Sargassum thunbergii* generally grew below *S. fusiformis*, except for the period from September to December, when *Chondria crassicaulis* grew dominantly. From the lower intertidal zone to the subtidal gentle slope at depths of up to 0.3 m, *Chondria ocellatus* and *C. verrucosus* grew dominantly from April to December. However, their growth zone was reduced to the lower

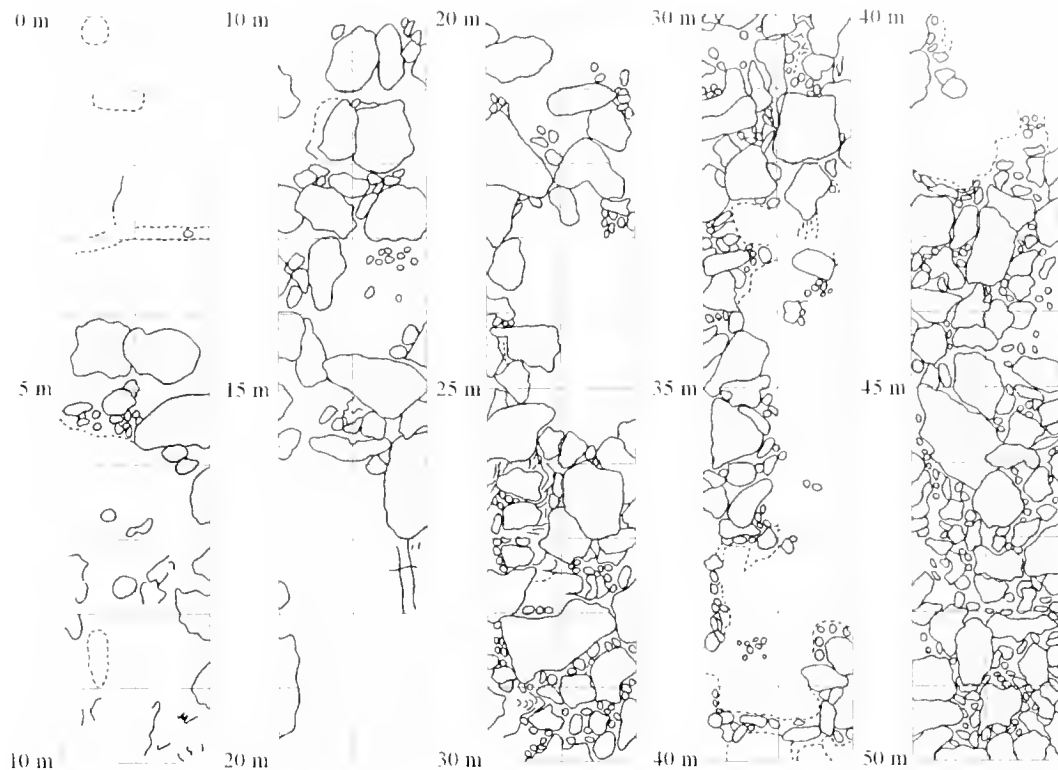


Figure 2. Bottom profile at the study site sketched using a  $1 \times 1$  m quadrat. Dashed lines represent rocks with ditches or holes.

intertidal zone in March and May the following year. From September to December, *Grateloupia livida* and *Acrosorium polyneurum* colonized the *Chondrus* growth zone. In March and May, *A. polyneurum* grew dominantly at a depth of 0–0.2 m. *Sargassum yezoense* and *S. micracanthum* grew on rocks at a depth of 0.3 m all year round. Those sargasso algae grew abundantly in June and July. After dieback, the holdfasts and stipes remained from September to March. At the deeper zone of >0.3 m, crustose corallines dominated, whereas *Dilophus okamurae* grew at a depth of 2.1–2.8 m from September to March and dominantly from October to January. The study site was characterized by depth, algal vegetation, and bottom character; the intertidal zone, the algal turf with gentle slope at the depth of 0–0.3 m, the upper steep-sloped crustose corallines at the depth of 0.3–1.0 m, and the lower steep-sloped crustose corallines at the depth of 1.0–2.8 m.

Except for the period from February to April and August, the water temperature during this study was markedly higher than the average. In particular, the deviation showed high values of 3.8°C in late December 1998 and 2.0–2.4°C in January and late April 1999 (Fig. 4).

#### Vertical Distribution

Seasonal changes in the density of *H. pulcherrimus* on the 4 characterized areas in the study are shown in Figure 5. Significant difference in the density among 4 areas ( $df = 3$ ,  $MS = 3951.505$ ,  $F = 163.587$ ,  $P < 0.0001$ ) and month ( $df = 9$ ,  $MS = 9.898$ ,  $F = 4.529$ ,  $P < 0.0001$ ) was found. Significant correlation between the area and month was also found ( $df = 27$ ,  $MS = 75.719$ ,  $F = 6.872$ ,  $P < 0.0001$ ). The densities on algal turf were significantly higher than those on the other areas throughout the year ( $P < 0.01$ ). There, the sea urchins were found beneath the boulders. Subse-

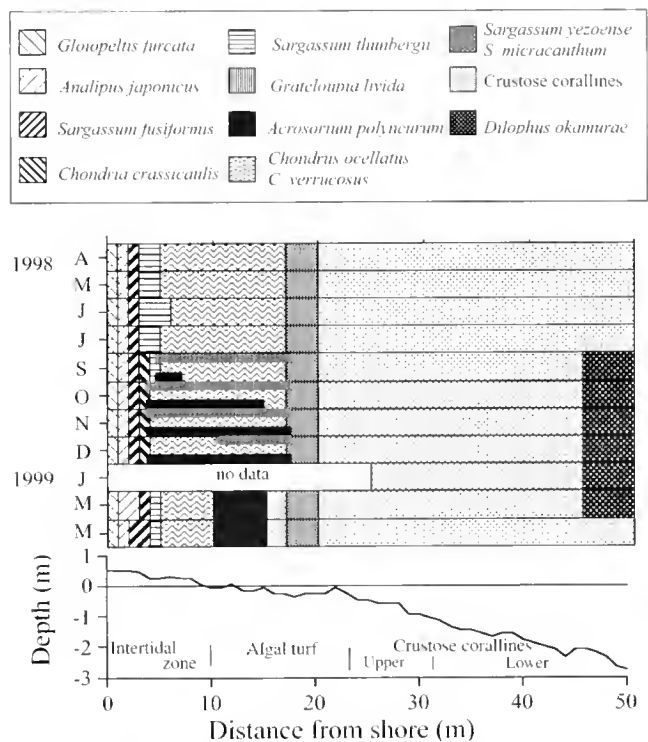


Figure 3. Depth and seasonal changes in the vegetation of the dominant algae with these coverage of ca. >50% at the study site characterized by the four areas, intertidal zone, algal turf, upper crustose corallines and lower crustose corallines.

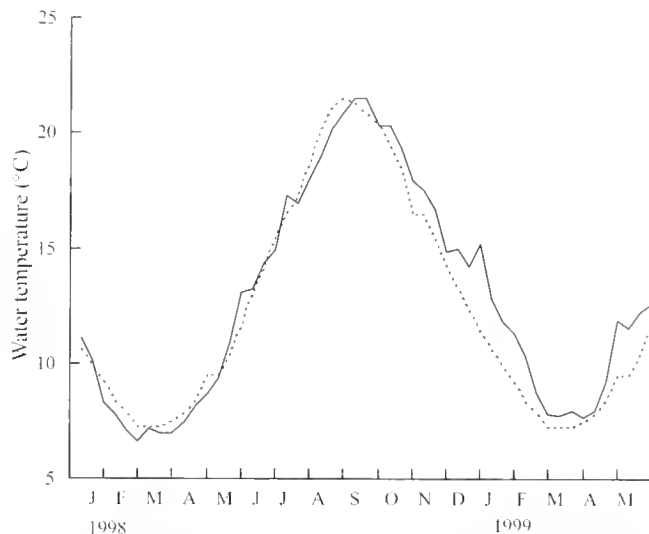


Figure 4. Surface water temperature during the study at Enoshima, near the study site during this study (solid line) and for 30 y average (dotted line) from 1968 to 1997.

quently, their densities on upper crustose corallines were significantly higher than those on intertidal and lower crustose corallines, with the exception of September 1998 and March and May 1999 ( $P < 0.01$ ).

The densities on the intertidal zone were low all year round. On algal turf, the densities from April to December 1998 ranged from 14.4–17.9 individuals/m<sup>2</sup>, and no significant difference was found ( $P > 0.05$ ). Then, the density significantly rose to 28.3 individuals/m<sup>2</sup> in March ( $P < 0.05$ ). In May, it was also higher than from April to November the previous year ( $P < 0.05$ ).

On the upper crustose corallines, the densities from November 1998 to January 1999 were significantly higher than those from May to September 1998 ( $P < 0.05$ ). In addition, those in December and January were higher than that in October ( $P < 0.01$ ). In particular, in January, the density reached a peak of 11.6 individuals/m<sup>2</sup>. Whereas, it significantly fell in March and May 1999, when it reached a peak on algal turf ( $P < 0.01$ ).

On the lower crustose corallines, the densities were low all year round. In January, the density reached a peak of 3.3 individuals/m<sup>2</sup>, significantly higher than that each month the previous year, with the exception of November ( $P < 0.01$ ). The density in November was higher than those in May, June, and September 1998 ( $P < 0.05$ ). The density in March and May 1999 was significantly lower than that in January ( $P < 0.01$ ), as observed on the upper crustose corallines. Thus a marked increase in the density of urchins on the algal turf from November to March was found.

The density on the algal turf in March 1999 was significantly higher than that in April 1998 ( $P < 0.01$ ), whereas that on the upper crustose corallines showed a significantly low value ( $P < 0.05$ ).

#### Size Frequency Distribution

Changes in the test diameter frequency distribution of *H. pulcherrimus* in each of the 4 areas are shown in Figure 6. At each area, sea urchins of 30–39 mm test diameter were predominant. On algal turf, the mode of test diameter distribution clearly shifted from 36–38 mm to 40–42 mm from June to December 1998. On the other hand, the sea urchins with 32–36 mm in test diameter increased and the mode shifted to 34–38 mm in March and May 1999, when the sea urchin densities rose significantly. On the

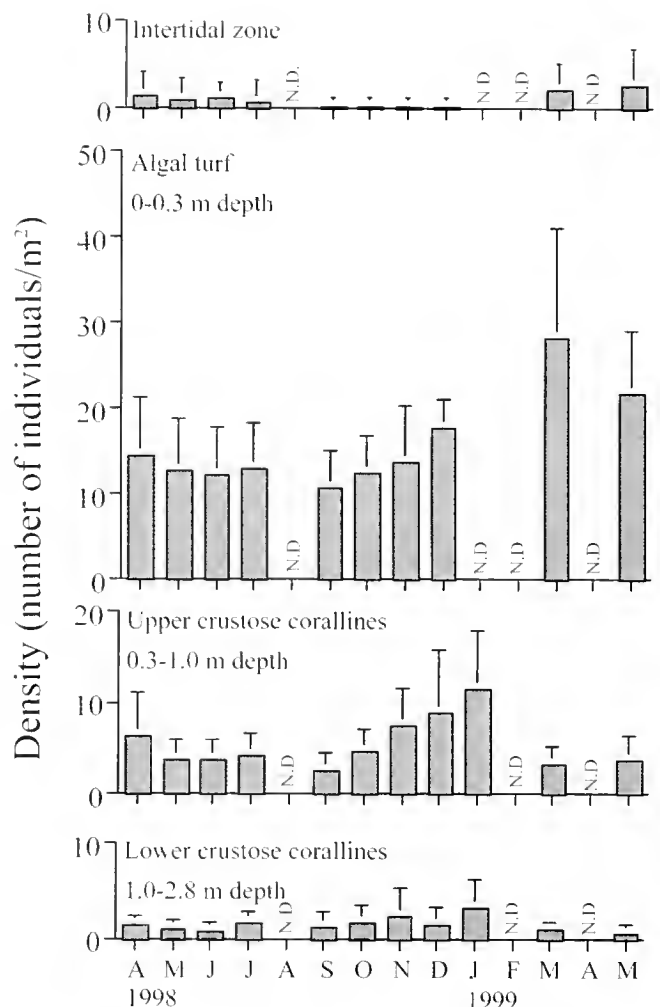


Figure 5. Seasonal changes in the density of *Hemicentrotus pulcherrimus* at the four areas, intertidal zone, algal turf, upper crustose corallines, and lower crustose corallines, at the study site. Vertical bars represent standard deviations. N.D. represents no data.

upper crustose corallines, the mode was around 40 mm from June to October, whereas urchins of 30–38 mm test diameter increased from November to January. Moreover, sea urchins of 32–36 mm test diameter were clearly found in each area in April and May 1998.

#### Gonad Development Process

The gonad development of the sea urchins was classified into five stages from histological observation as shown in Figure 7, and the seasonal changes on algal turf and crustose corallines were shown in Figure 8. From June to September, the gonads of the sea urchins at each algal community were in the recovering stage, with small numbers of oocytes and spermatocytes, with nutritive phagocytes filling the lumen (Fig. 7, 1A, 1B). Then, they progressed from the growing stage (Fig. 7, 2A, 2B), with increasing numbers of oocytes or spermatocytes along the acinal wall in October, to the mature stage (Fig. 7, 4A, 4B), in which most of the lumen is filled with ova and spermatozoa, in December or January. For long periods from January to June, the spent stage continued, with some relict ova and spermatozoa and empty spaces in the lumen (Fig. 7, 5A, 5B).

Seasonal changes in the gonad indices of the sea urchins at each



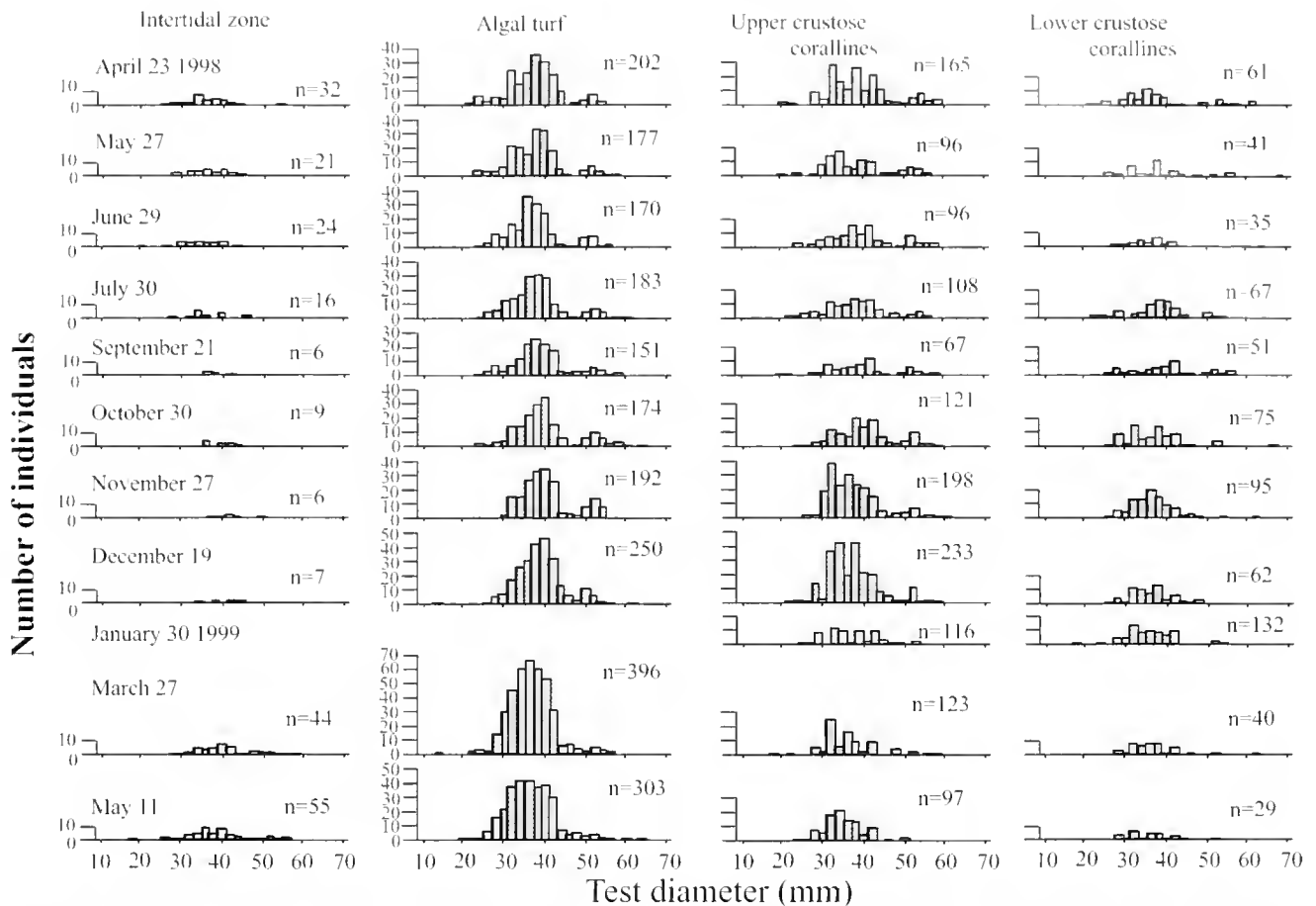


Figure 6. Seasonal changes in the test diameter frequency distribution of *Hemicentrotus pulcherrimus* at the four areas, intertidal zone, algal turf, upper crustose corallines, and lower crustose corallines, at the study site.

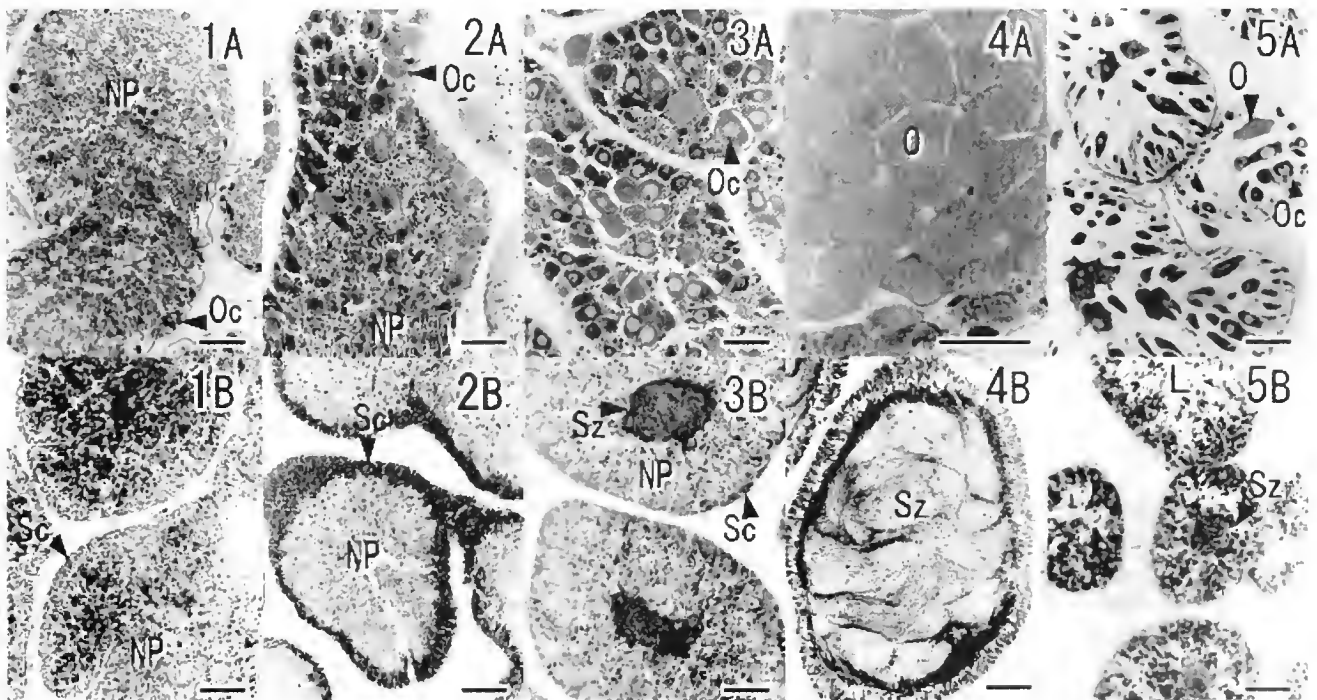


Figure 7. Histological identifications of the gonad developmental stages of *Hemicentrotus pulcherrimus*. Series A and B represent the developmental stages of ovary and testis, respectively. 1, recovering; 2, growing; 3, premature; 4, mature; 5, spent; Oc, oocyte; Np, nutritive phagocyte; O, ovum; Sc, spermatocyte; Sz, spermatozoa; L, lumen. Scale bars represent 100  $\mu$ m.

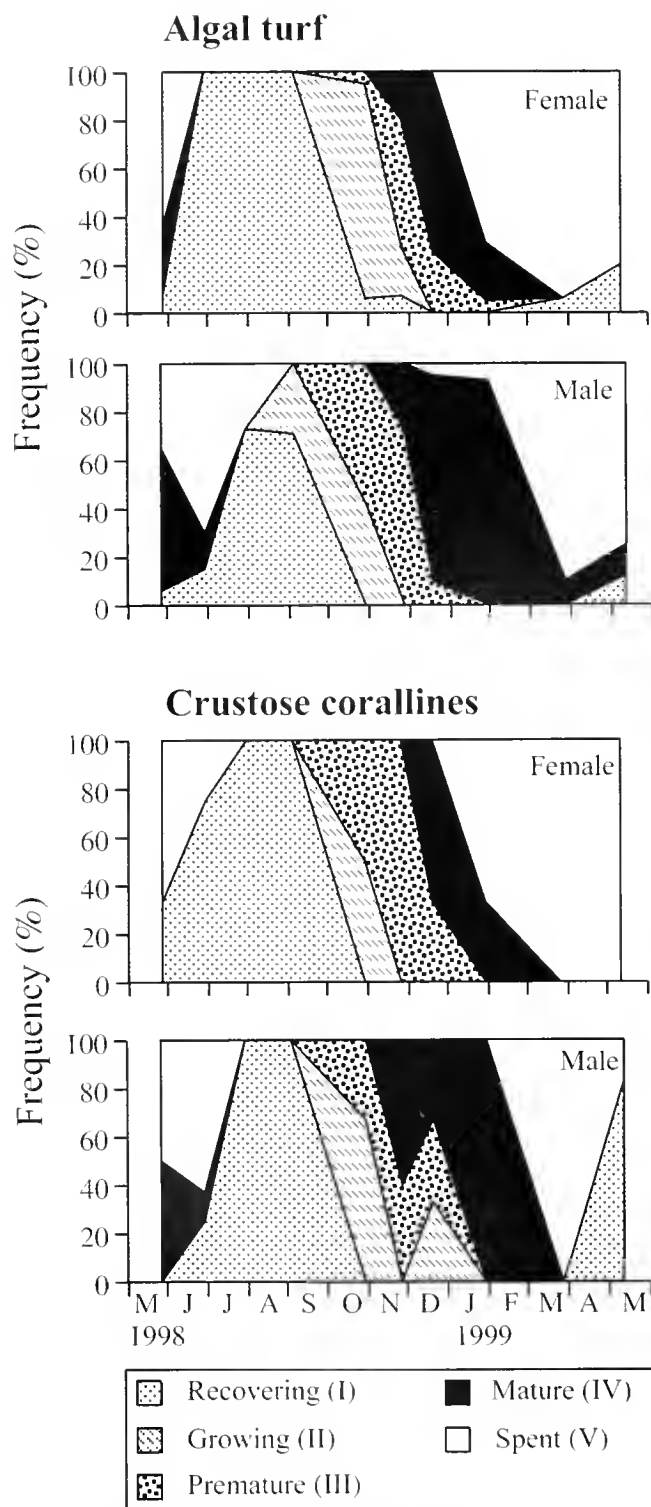


Figure 8. Seasonal changes in the gonad developmental stage of *Hemacentrotus pulcherrimus* at algal turf and crustose corallines.

community are shown in Figure 9. At each community, a significant seasonal difference in gonad indices was found (Algal turf:  $df = 9$ ,  $MS = 371.4$ ,  $F = 35.956$ ,  $P < 0.0001$ , Crustose coralline:  $df = 9$ ,  $MS = 32.1$ ,  $F = 3.968$ ,  $P < 0.0026$ ). There was no statistical difference in the gonad indices at algal turf from May to

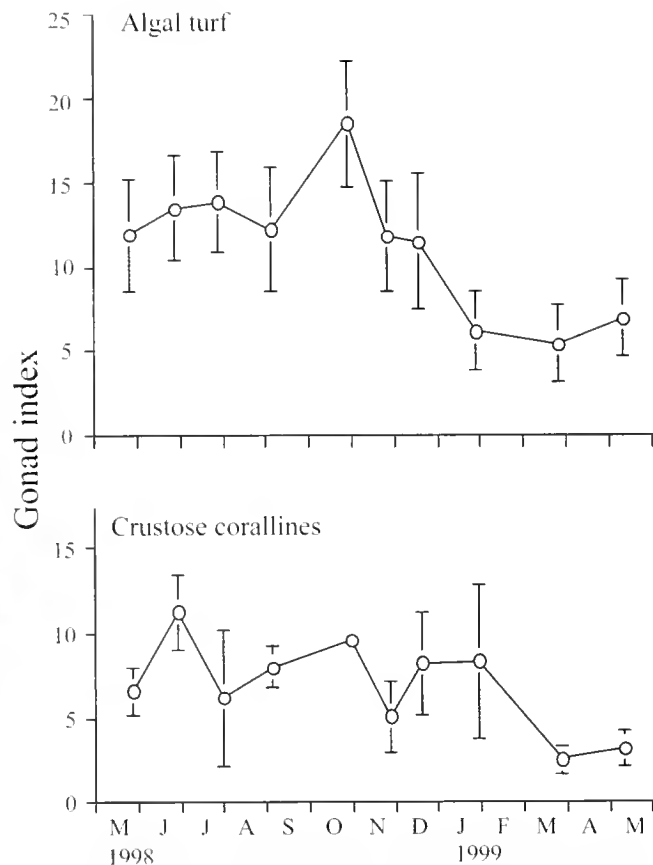


Figure 9. Seasonal changes in the gonad indices of *Hemacentrotus pulcherrimus* at algal turf and crustose corallines. Vertical bars represent standard deviations.

September 1998, when those ranging from 11.9–13.8 were in the recovering stage ( $P > 0.05$ ). Then, the indices significantly rose to 18.5 in October, when gonad development was in the growing and premature stages ( $P < 0.01$ ). The indices significantly fell to the minimum value of 5.4 in March 1999 ( $P < 0.05$ ) and remained at that value until May. The gonad indices on crustose corallines ranged from 5.0–11.2 from May 1998 to January 1999, and no seasonal difference in the indices was found ( $P > 0.05$ ). In March, the indices showed a minimum value of 2.5, similar to those found on algal turf. Except for June, December, and January, the gonad indices on algal turf were higher than those at crustose corallines ( $P < 0.05$ ).

#### Gut Contents

The gut contents of *H. pulcherrimus* at each algal community are identified as shown in Table 1. In the gut contents of sea urchins on algal turf, the algae of 15 species, 7 genera and 2 unidentified taxa were found. These were small algae, except for *Sargassum* spp. The seagrass *Zosteraceae*, the attached diatom *Bacillariophyceae*, a terrestrial plant, and animals such as *Gastropoda*, *Bivalvia*, and *Crustacea* were also found. In those components, *Sargassum* spp., *Chondrus* spp., *Acrosorium polynurum*, and *Crustacea* occurred all year round. On crustose corallines, a similar number of species, genera and taxa of algae occurred despite the fact that few algal species grow, and the other compo-

TABLE 1.

Gut contents of *Hemicentrotus pulcherrimus* at algal turf (open circles) and crustose corallines (filled circles).

Species	1998							1999		
	May	Jun.	Jul.	Sep.	Oct.	Nov.	Dec.	Jan.	Mar.	May
<i>Ulva pertusa</i>	●	○	○							
<i>Cladophora conchophoria</i>			○							
<i>Codium hubbsii</i>		●	●	●						
<i>Ralfsia</i> spp.		●	●		●	●	●	●	●	●
<i>Dictyota dichotoma</i>				●	○	○	●	○		
<i>Dilophus okamurae</i>		●	●	●	●	●	●	●	●	●
<i>Elachista okamurae</i>			●							
<i>Sargassum</i> spp.	●	●	○	●	●	●	●	○	●	●
Crustose corallines	●	●	●	●	●	●	●	●	●	●
<i>Corallina</i> spp.									●	
<i>Gelidium elegans</i>	●	●	●	●			●	●	●	●
<i>Chondrus</i> spp.		●	●	●	●	●	●	●	●	●
<i>Carpopeltis</i> spp.	●		●							
<i>Hypnea</i> spp.		●								
<i>Amphileptopsis flabelliformis</i>	●	●								
<i>A. paradoxa</i>		●								
<i>Plocamium telfairiae</i>		●	●	●		●	●	○	○	
<i>Lomentaria catenata</i>	○	●	●			●	●	○	○	●
<i>L. hakodatensis</i>		●				●		●	●	
<i>Heterosiphonia pulchra</i>	●	●				●				
<i>Acrosorium polyneurum</i>	●	●	●	●	●	●	●	●	○	○
<i>Laurencia</i> spp.			●	●						
<i>Polysiphonia</i> spp.					○					
<i>Symphocladia latiuscula</i>		●			○	○	○		○	○
<i>S. marchantioides</i>			●			●				
Zosteraceae	○	●	●	●	○	●	●	●	●	●
Bacillariophyceae										
Terrestrial plant	○		●		●		●	●		●
Hydrozoa		●								
Bryozoa	○		●	●	●					
Gastropoda			●	●						
Eumalacostraca	○	●	●		○				○	
Crustacea	○	○	●	●	○	●	●	●	○	○
Feather of terrestrial insect							●			
Feather of bird		○								
Plastic		○					●	●	○	
Others <sup>a</sup>	○	●	●	●	○	●	●	●	○	○

<sup>a</sup> Residual matters including the unclassified plants and animals, and sand.

nents were similar to those found on algal turf. Crustose coralline, *A. polyneurum*, and Crustacea occurred all year round.

The seasonal changes in the mean weight percentage of each of the 6 main algal components to total gut contents are shown in Figure 10. On algal turf, *Chondrus* spp., *A. polyneurum*, and *Sargassum* spp. showed high percentages, reflecting the dominant vegetation. The percentage of *Chondrus* spp. was high from July to October. From November to March, the percentage of *A. polyneurum* ranged 30% to 40%, higher than that of five other algae. In May 1999, the percentage of *Sargassum* spp. abruptly rose to 49%, in contrast to fall in that of *A. polyneurum*. On crustose corallines, a high percentage of crustose coralline was found all year round; particularly, the percentage reached 49% to 78% from October to May. In June, July and September, that percentage fell to <21%. In June, the percentage of *Sargassum* spp. abruptly rose to 42%, higher than that of other algae. In September, *C. hubbsii* also

showed high percentage. The percentage of crustose coralline in May 1999 was higher than that in May 1998.

## DISCUSSION

The densest habitat of *H. pulcherrimus* was found on algal turf at a depth of 0–0.3 m, where boulders dominated. Similarly, a dense distribution beneath boulders in shallow waters is found in other regions in Japan (reviewed by Agatsuma 2001a). However, their low density on crustose corallines at a depth of >1.0 m with boulders shows that the distribution of *H. pulcherrimus* is not restricted by the factor of bottom characteristics alone. The seasonal changes in density suggest that the sea urchins migrate from crustose corallines to algal turf from November to March. Juvenile *H. pulcherrimus* with 0.8–4.5 mm in test diameter were observed in *Amphiroa dilatata* and the roots of *Phyllospadix japonicus* in shallow waters of the Japan Sea, Fukui (Kawana 1938). However,

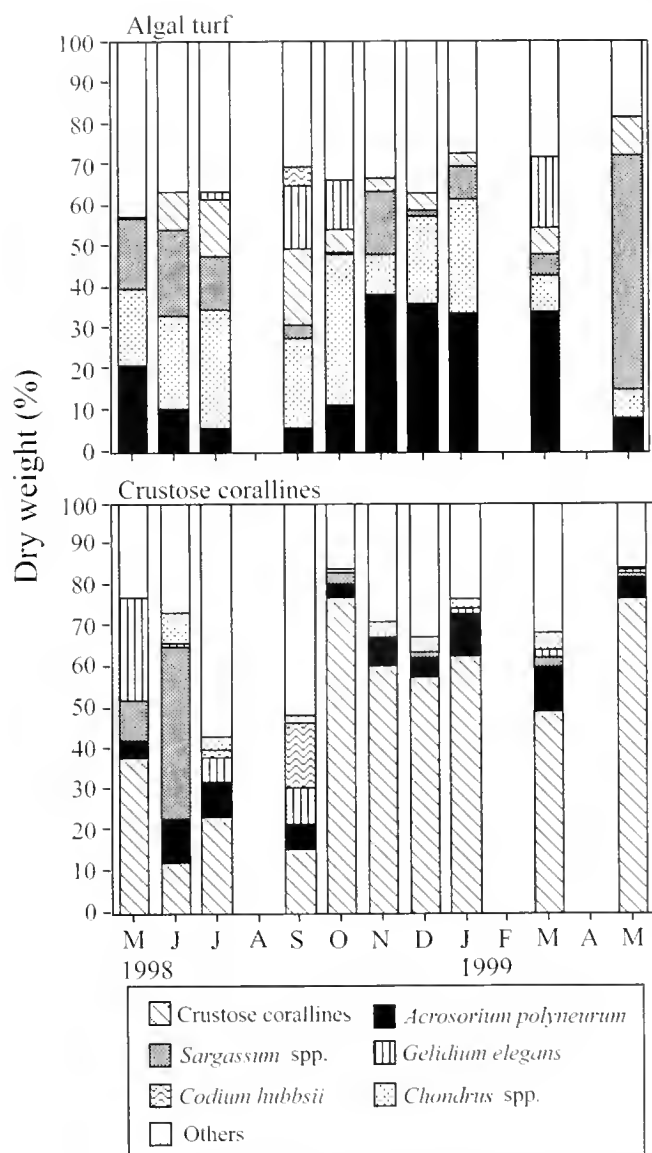


Figure 10. Seasonal changes in the mean weight percentage of each of six algal species as the main components in the gut contents of *Hemicentrotus pulcherrimus* at algal turf and crustose corallines.

it is unknown where the larvae settle and metamorphose. The migration from crustose corallines raises the possibility that the larvae settle on crustose corallines, as do *Strongylocentrotus purpuratus* and *S. franciscanus* (Cameron & Schroeter 1980, Rowley 1989), *S. nudus* (Taniguchi et al. 1994, Sano et al. 1998), and *S. droebachiensis* (Balch & Scheibling 2000).

The gonads of *Strongylocentrotus* spp. in a kelp forest grow larger than they do in barren grounds (Agatsuma 2001b, Gonor 1973, Lang & Mann 1976, Pearse 1980, Wharton 1980, Johnson & Mann 1982, Keats et al. 1984, Scheibling & Stephenson 1984, Sivertsen & Hopkins 1995, Meidel & Scheibling 1998). The difference in gonad mass is caused by food availability at each habitat (Lang & Mann 1976, Sivertsen & Hopkins 1995). Meidel & Scheibling (1998) clarified the difference between the food availability of *S. droebachiensis* in kelp (*Laminaria longicruris*) forest and barren grounds from quantitative gut content analysis.

In the present study, gut content analysis showed that the fo-

liose algae *Chondrus* spp., *A. polyneurum*, and *Sargassum* spp. were main foods for *H. pulcherrimus* on algal turf. The high percentage of crustose coralline in the gut contents of the sea urchins on crustose corallines results in lower gonad indices than those on algal turf. From changes in size frequency distributions, seasonal growth was clearly found from June to December. In the gut contents of the sea urchins on crustose corallines during this period, the percentage of crustose coralline was lower than that in other months, as *Sargassum* spp., *Codium hubbsii* and the other algae were supplied as drift algae from shallow waters. It is more likely that those drift algae contribute the growth of the sea urchins on crustose corallines. The down-shift in the modal size of sea urchins on the algal turf during the migration period could be attributed to the immigration of smaller, low-growing ones from crustose corallines in deeper waters. In addition, the growth of sea urchins on algal turf is considered to be mainly supported by *Chondrus* spp., which showed a high weight percentage in the gut contents until October.

The histological observation of gonad and the seasonal changes in gonad indices show that spawning occurs from January to May, reaching a peak from January to March, as it does in other regions in western Japan (Agatsuma 2001a). Gonad size reached a peak corresponding to maturation and spawning from October to March. The rise in the weight percentage of crustose coralline, which was more than approximately 50% in the gut contents of the sea urchins on crustose corallines after September, is related to a decrease in the supply of drift algae from shallow waters. Therefore, these findings suggest that the grazing activity switched to an active and mobile mode, as found in *S. purpuratus* and *S. franciscanus* (Mattison et al. 1977, Russo 1979, Harrold & Reed 1985) and *Evechinus chloroticus* (Andrew & Stocker 1986), and the movement to algal turf in shallow waters was triggered. As *A. polyneurum* grew dominantly from October to March and showed a high percentage in the gut contents, this alga can be considered to be food that ensures gonad development and success in the reproduction of the sea urchins that migrate from crustose corallines to algal turf. Thus, it is suggested that the migration is related to the search for the algal food *A. polyneurum*.

In the meanwhile, the density of *H. pulcherrimus* on algal turf in March 1999 was higher than that in April and May 1998. In addition, the percentage of crustose coralline in the gut contents on crustose corallines in May 1999 was significantly higher than that in May 1998. These results may suggest that much higher water temperatures during winter in 1999 than those of average years reduced the algal communities as found in Oshika Peninsula near the study site in 1980s (Taniguchi 1991), causing algae to subsequently drift, and resulted in the migration of many sea urchins with poor gonad development to shallow waters. For the sympatric sea urchin *S. nudus*, no 0–1-y-old juveniles with a food habit of attached diatoms and small algae migrate from crustose corallines, where larvae settle, to a kelp (*L. religiosa*) forest (Agatsuma 2001b). In the present study, the pattern of migration of juvenile *H. pulcherrimus* is unclear, as few juveniles of test diameter less than 10 mm were observed. Maturation size is considered to be at 26 mm in test diameter in Fukui (Kawana 1938). Whereas, the difference in maturation size among localities and maturation age is unknown. The changes in test diameter distribution may suggest that over 2-y-old urchins with a test diameter of 32–36 mm were the ones that mainly migrated. Thus, it is unclear whether those migrating sea urchins correspond to the first maturation-size group.

After the migration of *H. pulcherrimus*, no return to crustose corallines was observed. The residence of *H. pulcherrimus* on algal turf is likely to be related to food supply, but it additionally ensures a suitable habitat beneath boulders, which reduce the action of the waves. In addition, a study on the morphological characteristics with thick test in relation to attachment strength of the foot tube, and tolerance of test and spine from abrasion would contribute to understanding their adaptation to a habitat in shallower waters. In the areas in Yamaguchi and Kanagawa Prefectures where *H. pulcherrimus* is observed, the large brown algae Sargassaceae and Laminariales grow sympatrically (Agatsuma 2001a). The interactions between specimens of *H. pulcherrimus* and the marine forest should be studied seasonally.

Significant gonad enhancement of *H. pulcherrimus* on algal turf was found from September to October. The maximum gonad index of 18.5 in October coincides with that of 17–19 at the fishing grounds in Saga, where the most rapid growth in western Japan was reported (Ito et al. 1989, Shimazaki et al. 1987), although

lower than that of 25 in Oshoro Bay, Hokkaido (Agatsuma & Nakata 2004). The seasonal growth and gonad enhancement on algal turf are likely to be caused by *Chondrus* spp., which grew dominantly and showed a high percentage in gut contents. Therefore, the dietary effect of *Chondrus* spp. and *A. polyneurum* as the main food for migrating sea urchins on growth and gonad development should be evaluated by means of a rearing experiment.

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## ADAPTATION OF RAY'S FLUID THIOLYCOLLATE MEDIUM ASSAY TO DETECT AND QUANTIFY PLANKTONIC STAGES OF *PERKINSUS* SPP. PARASITES

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**ABSTRACT** The parasites *Perkinsus* spp. are relatively easily and inexpensively detected in host tissues, but available methods to detect free-living planktonic stages are technologically complex and expensive. As a result, few studies have been conducted to detect and quantify free-living stages during transmission. Here, we describe an adaptation of Ray fluid thioglycollate medium (RFTM) assay to detect and enumerate *Perkinsus* spp. parasites in environmental water samples. Recovery of *in vitro* cultured *P. marinus* was successful, but recovery rates were low. Filtration of water samples captured significantly more cultured *P. marinus* cells than centrifugation. Lipid supplementation during RFTM incubation enhanced recovery of cultured *P. marinus*, but not of naturally occurring *Perkinsus* spp. parasites. Comparisons between the modified RFTM filtration method and a more complex immunoassay revealed that the two methodologies were equally sensitive, indicating that the RFTM filtration method may be confidently applied to environmental water samples to determine relative concentrations of *Perkinsus* spp. parasites.

**KEY WORDS:** *Perkinsus marinus*, dermo, disease, oyster, RFTM, planktonic, filtration

### INTRODUCTION

The protozoan parasite *Perkinsus* that are pathogenic to a variety of shellfish worldwide (Bower et al. 1994). Most notable in North America is *Perkinsus marinus* Levine, a protozoan endoparasite that causes dermo disease in the eastern oyster, *Crassostrea virginica* Gmelin. The parasite is common from Maine to Mexico (Krantz & Jordan 1996) and is responsible for mass oyster mortalities in Delaware Bay, Chesapeake Bay, and the Gulf of Mexico (Ford & Tripp 1996). It has long been known that direct transmission of *P. marinus* to *C. virginica* occurs via the water column (Ray 1954), but few attempts have been made to develop and apply methods to detect *P. marinus* in the water column. As a result, knowledge about planktonic stages of *P. marinus* and the processes that affect this important life cycle phase is limited. Applications of an immunoassay (Dungan & Roberson 1993) and the polymerase chain reaction (PCR) in combination with species-specific molecular genetic probes to quantify planktonic stages in environmental water samples have met with varying success (Roberson & Dungan 1994, Yarnall 1998, Ragone Calvo et al. 2003, Audemard et al. 2004). These methods are technologically complex, not readily available to many laboratories and expensive. This study describes the adaptation of Ray fluid thioglycollate medium (RFTM) assays for enumerating planktonic stages of *Perkinsus* spp. parasites from environmental water samples.

Since the discovery of *P. marinus* in the 1940s (Mackin et al. 1950), detection and enumeration methods have focused on categorizing infections within oyster tissue. These methods include histology, tissue smears, the RFTM assay, a polyclonal immunoassay and various molecular genetic probes. Tissue smears and histological methods that were initially used to diagnose infections (Mackin et al. 1950) were soon replaced by the RFTM assay (Ray 1952, 1966). During this assay, the parasite enlarges and develops a thick cell wall that stains blue-black with Lugol's iodine, facilitating its detection. The RFTM assay is easy to perform, inexpensive and sensitive when combined with NaOH digestion of the sample (Choi et al. 1989, Bushek et al. 1994). It is not species-

specific, but it appears to be genus-specific and remains the most commonly used method for detection of *Perkinsus* spp. infections in molluscan hosts (Bower & McGladdery 2003).

The RFTM assay has been adapted to quantify *P. marinus* in tissues (Choi et al. 1989), hemolymph (Gauthier & Fisher 1990), and entire individuals (Bushek et al. 1994, Fisher & Oliver 1996). Of particular relevance to quantifying *P. marinus* and other *Perkinsus* spp. in plankton is the discovery by Choi et al. (1989) that oyster tissues, bacteria and most other contaminants may be digested away with 2M NaOH leaving a suspension of parasites (actually only the cell walls) that may be stained and counted. Fisher and Oliver (1996) demonstrated that NaOH digestion does not affect the thick cell wall that *P. marinus* develops during RFTM incubation. Two assumptions, however, must be made to use the RFTM assay quantitatively. First, all life stages are assumed to enlarge during incubation and to stain with Lugol's iodine for detection (Ray 1952). Early comparisons between the RFTM method and histology revealed that all identifiable stages of *P. marinus* occurring within host oysters enlarged in RFTM (Ray 1954, Stein & Mackin 1957). Additional support for this assumption was provided when Ragone Calvo and Bureson (1994) were unable to detect any previously unknown stages of *P. marinus* with a polyclonal anti *P. marinus* antiserum applied to histological samples. The second assumption is that no proliferation occurs during incubation in RFTM. By monitoring *P. marinus* counts and enlargement throughout incubation in RFTM, Ray (1952) and Stein and Mackin (1957) provided evidence to support this assumption. In addition, extensive efforts to culture *P. marinus* continuously in RFTM were unsuccessful (Ray 1954, Prokop 1950, Mackin & Boswell 1956). Under these assumptions, NaOH digestion of RFTM incubated tissues is routinely used to quantitatively enumerate *P. marinus* in many laboratories.

We applied RFTM incubation and NaOH digestion to environmental water samples as a method to enumerate planktonic stages of *P. marinus*. After initial attempts yielded positive ( $\leq 5$  cells/liter) results, a series of experiments was conducted to determine the efficiency of the method and to identify possible improvements. The goal was to develop a simple, inexpensive method to quantify *P. marinus* from environmental water samples using tools

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generally available to laboratories capable of performing standard RFTM assays.

## METHODS AND RESULTS

Five experiments were conducted sequentially to examine various aspects of the assay. The first three experiments were performed with *in vitro* cultured *P. marinus* to determine the efficiency of and possible improvements to the method, whereas the latter two experiments examined naturally occurring *P. marinus* in environmental water samples. Because results of each experiment were used in designing subsequent experiments, methods and results are presented together for each experiment. All data were analyzed using SYSTAT statistical software (Wilkinson 1998).

### Experiment 1

#### Centrifugation Versus Filtration

This experiment was designed to determine if there was a difference in recovery of parasite cells collected via centrifugation or filtration. Replicate samples (500 mL) of 1- $\mu$ m filtered seawater (FSW) were spiked with 1-mL aliquots of *in vitro* cultured *P. marinus* cells that had been maintained in ATCC 1886 medium (www.atcc.org) with minor modifications described in Bushek et al. (2000). After mixing the parasites in each sample, they were recovered as pellets via centrifugation ( $\times 650$  g,  $\times 2603$  g, or  $\times 5858$  g for 15 min,  $n = 2$  per treatment) or filtered (vacuum pressure = 175 mm Hg,  $n = 3$ ) onto a Gelman GN-Metricel 0.45- $\mu$ m membrane. Pellets and filters were transferred to 15-mL conical centrifuge tubes containing 10 mL RFTM fortified with 10  $\mu$ L of penicillin-streptomycin (Sigma P-0781) to retard bacterial growth. After incubation at room temperature for one week in the dark, the samples were pelleted at  $\times 823$  g for 15 min. Centrifuged samples were resuspended in 5 mL 0.2- $\mu$ m FSW, stained with Lugol's iodine and placed on a Gelman GN-Metricel Membrane filter paper (0.45  $\mu$ m pore size) under 100 $\times$  magnification for enumeration (Fisher & Oliver 1996). Filtered samples were resuspended in 2M NaOH (Choi et al. 1989) to digest the filter (2 h at 60  $^{\circ}$ C), then washed twice in 0.2- $\mu$ m FSW, resuspended in 5 mL 0.2- $\mu$ m FSW, stained with Lugol's iodine and enumerated with a Hausser counting chamber under 100 $\times$  magnification. The enumeration of filtered samples differed from centrifuged samples because small portions of the filter that remained after digestion obscured the parasites when counted on a Gelman GN-Metricel Membrane filter. The Hausser counting chamber slowed processing because samples were no longer coplanar, but eliminated the problem of filter particulates obscuring parasites. Enumerating all parasites in these samples required focusing through layers of the medium.

*In vitro* cultured *P. marinus* were recovered and successfully enumerated from all centrifugation and filtration samples. The parasites were more difficult to detect than expected because, compared with parasites in oyster tissues, relatively little enlargement occurred during RFTM incubation, and staining with Lugol's iodine was weak. Although the initial concentration of parasites used to obtain initial aliquots had not been precisely determined, the percentage of cells recovered was much lower than expected, based on earlier cell counts from the *in vitro* cultures. Unfortunately, this oversight precluded an estimate of recovery efficiency. Nevertheless, because equal aliquots from the same parasite suspension were used, it was clear that significantly more parasites were consistently recovered with filtration than with centrifugation

treatments (Tukey,  $P < 0.001$ , Fig. 1). Centrifugation recovery decreased with increasing centrifugal force;  $\times 650$  g centrifugation resulted in a significantly higher parasite recovery than  $\times 5858$  g centrifugation (Tukey,  $P = 0.018$ ), but recovery at the intermediate velocity did not differ significantly from either extreme. Logistically, centrifugation was more time consuming than filtration, requiring 45 min to concentrate samples compared with 15 min for filtration. Because filtration was more efficient than centrifugation, subsequent experiments used the filtration method only.

### Experiment 2

#### Recovery Efficiency

This experiment involved a series of treatments designed to measure recovery efficiency and identify potential sources of parasite loss. In addition, because enlargement and staining were lower than expected in Experiment 1, each treatment was duplicated with RFTM that was supplemented 1:100 (v/v) with lipid concentrate (+lipids). Nickens et al. (2002) reported that the addition of lipids may increase enlargement and cell wall development of *in vitro* cultured *P. marinus* during RFTM incubation. The lipids used for supplementation (GIBCO BRL 21900-030) were derived from cod liver oil and supplemented with cholesterol, Pluronic F-68, DL- $\alpha$ -tocopherol acetate and Tween 80. Treatment 1 (water + filter + RFTM) and treatment 2 (water + filter + RFTM + lipids) replicated the entire RFTM filtration methodology used in Experiment 1, except that samples consisted of only 200-mL FSW and were spiked with known concentrations of viable cultured *P. marinus* clones ( $4.04 \times 10^5$  cells of isolate LA22 clone 8,  $5.64 \times 10^5$  cells of isolate LA25 clone 2, and  $3.97 \times 10^5$  cells of isolate LA25 clone 4; These isolates were derived from oysters collected from the Gulf coast of Louisiana, USA and provided by J LaPeyre). In treatments 3 (filter + RFTM) and 4 (filter + RFTM + lipids), parasites were added directly to the filter without applying a vacuum. These treatments tested the effects of handling the parasites (i.e., moving parasites from culture medium into seawater and capturing them on the filter). In treatments 5 (RFTM) and 6 (RFTM + lipids), parasites were added directly to RFTM to determine if the filter or NaOH digestion interfered with enlargement and detection of the parasites. Because no filter was used in treat-

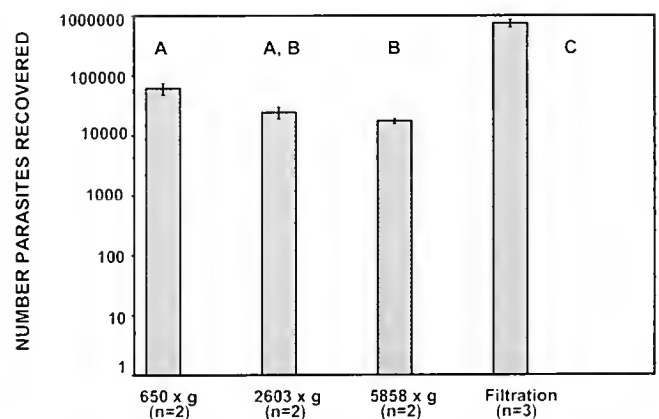


Figure 1. Centrifugation ( $\times 650$  g,  $\times 2603$  g, and  $\times 5858$  g) versus filtration (vacuum pressure = 175 mm Hg) of water samples for recovery and enumeration of *P. marinus*. Error bars represent one SEM. Treatments with the same letter are not significantly different at  $\alpha = 0.05$ .



ments 5 and 6, there was no need to perform the NaOH digestion step.

The percentage of parasites recovered was calculated for each treatment then arcsine transformed to normalize the data for analysis of variance (Sokal & Rohlf 1981). The variables filter, water and lipids were examined for differences in recovery. A treatment involving water but no filter would have completed a 3-way ANOVA; however, this treatment was not possible to test without centrifugation, which would have confounded the experiment. The absence of this treatment resulted in lost degrees of freedom for the 3-way ANOVA. Therefore, the 3-way interaction between filter, water and lipids; and the 2-way interaction between filter and water, were removed as suggested for a fractional factorial design in which data cells are missing (Wilkinson 1998).

The recovery efficiency of the RFTM filtration method used in Experiment 1 (treatment 1) was surprisingly low:  $2.71\% \pm 0.42$  (Table 1). Lipid supplementation resulted in significantly higher recovery across all treatments (Table 2, Fig. 2). The addition of lipids increased the number of parasites detected and generally increased the intensity of staining, but did not increase enlargement of the cells. The ANOVA in Table 2 indicated a significant effect of adding the cultured cells to water before capturing them on a filter. Inspection of Figure 2 indicates that treatments 1 and 2, when parasites were first diluted in FSW, resulted in considerably lower recovery than their respective treatments (3 and 4) when parasites were added directly to filters. Recovery was intermediate when parasites were added directly to RFTM (Fig. 2). Because recovery for treatments 5 and 6 was not greater than recovery for other treatments, cell loss was not attributed to effects of NaOH digestion.

### Experiment 3

#### Lipid Dose Effects

This experiment was designed to determine if a higher concentration of lipids would further enhance recovery of *in vitro* cultured *P. marinus*. Nine, 200-mL samples of FSW were spiked with known concentrations of viable cells from one of three discrete *P. marinus* isolates ( $10.2 \times 10^5$  cells of ATCC 50768;  $7.53 \times 10^5$  cells of ATCC 50889 and  $4.57 \times 10^5$  cells of ATCC 50776). Lipid dose treatments included no lipids (control), 1:100 (v/v) lipids/RFTM, and 1:10 (v/v) lipids/RFTM. Lipids were added to RFTM prior to sample incubation. Three replicate samples were tested per treatment and a different *P. marinus* isolate was used for each replicate to ensure results were not isolate specific. Water samples were filtered and processed following the procedure outlined in Experi-

TABLE 1.

Recovery efficiency of the RFTM filtration method. Recovery rates for Experiment 2, Treatment 1. The recovery rate for each replicate is indicated.

<i>P. marinus</i> Isolate	<i>P. marinus</i> Added	<i>P. marinus</i> Recovered	Percent Recovery
LA22 - 8	$40.4 \times 10^4$	$1.4 \times 10^4$	3.467
LA25 - 2	$56.4 \times 10^4$	$1.5 \times 10^4$	2.661
LA25 - 4	$39.7 \times 10^4$	$8.0 \times 10^3$	2.031
Mean $\pm$ (SEM)	$45.5 \times 10^4$ $\pm (54.4 \times 10^2)$	$1.2 \times 10^4$ $\pm (2.2 \times 10^3)$	2.71% $\pm (0.42)$

TABLE 2.

ANOVA results for Experiment 2. Asterisk and hold indicate significant effects at  $\alpha = 0.05$ . df = degrees of freedom, SS = sum of squares, MS = mean squares.

Source of Variation	df	SS	MS	F-Ratio	P-Value
Water*	1	0.254	0.254	5.707	<b>0.034</b>
Lipids*	1	0.229	0.229	5.137	<b>0.043</b>
Filter	1	0.154	0.154	3.462	0.087
Water $\times$ Lipids	1	0.025	0.025	0.558	0.469
Lipids $\times$ Filter	1	0.002	0.002	0.055	0.818
Error	12	0.534	0.045		

ment 2, treatment 1. After recovery rates were calculated, a 1-way ANOVA and a control versus lipid contrast were used to examine lipid dose effects.

Results showed that both lipid dosage treatments recovered more parasites than the control (Fig. 3), but the increase was not significant (1-way ANOVA,  $P = 0.555$ ). A contrast of control versus lipid treatments was also not significant ( $P = 0.346$ ). Although not significant, recovery was lower for the higher lipid concentration. Recovery in the 1:100 treatment was similar to treatment 2 in Experiment 2. That is, variability within treatments was high and may be attributed to lipid addition, differences between the *P. marinus* isolates, or both. Additional experiments that include more isolates than were available at the time of these experiments are needed to resolve the source of variation.

### Experiment 4

#### Environmental Water Sample Enumeration and Lipid Dose Effects

This experiment was essentially identical to Experiment 3, but the methods were applied to environmental water samples rather than *in vitro* cultured *P. marinus*. Three environmental water samples (500-mL each) were collected August 22, 1998 from Oyster Landing, North Inlet, South Carolina using two automated

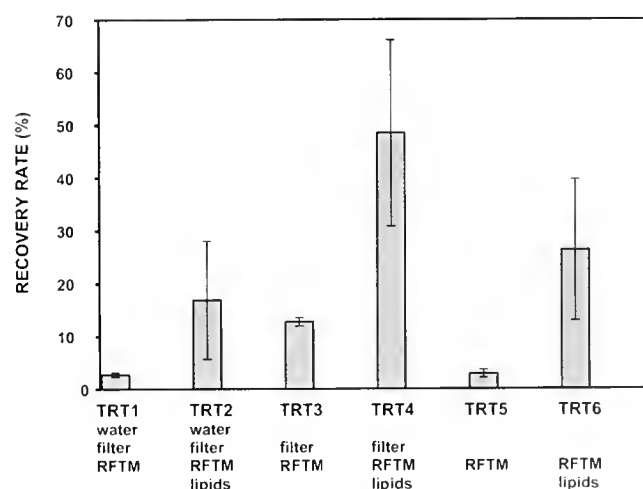


Figure 2. *Perkinsus marinus* cell recovery for treatments in Experiment 2. Parasites were diluted in FSW then filtered in treatments 1 and 2, added directly to filters for treatments 3 and 4, and added directly to RFTM for treatments 5 and 6. A 1% lipid supplement (1:100 v/v) to RFTM was added to Treatments 2, 4, and 6. Data are means  $\pm$  SEM of three replicates.

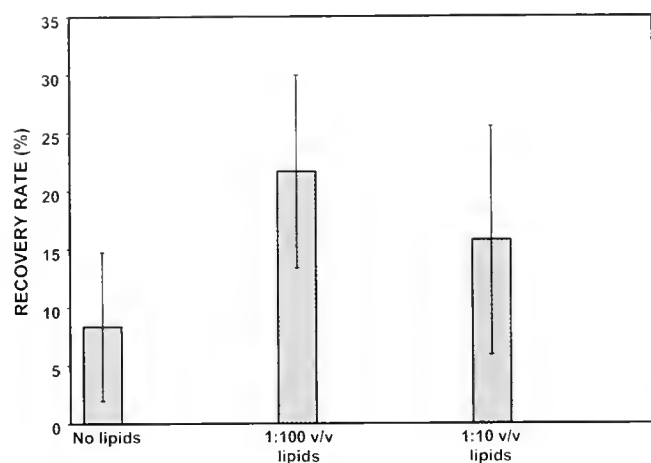


Figure 3. Lipid dose effects on cultured *P. marinus* cell recovery. Three lipid concentrations were tested: control = no lipids, 1:100 (v/v) lipids/RFTM, and 1:10 (v/v) lipids/RFTM. Data are means  $\pm$  SEM of three discrete clones for each treatment. No significant differences existed among treatments ( $P = 0.555$ ).

ISCO water samplers. Each sample was prefiltered through a 25- $\mu$ m nylon mesh, divided into three equal aliquots, and then processed as described for Experiment 3 using the same lipid treatments. Because *P. marinus* is the only species of *Perkinsus* that has been documented to occur in South Carolina (Reece et al. 1997, 2001), all cells positive per the RFTM assay were considered to be *P. marinus*. Counts were standardized to parasites per liter and then  $\log_{10}$ -transformed to normalize the distribution for a 1-way ANOVA (Sokal & Rohlf 1981). Tukey multiple comparisons were used to differentiate significance levels among treatments, and a control versus lipid contrast was calculated to test the effect of adding lipids.

Unambiguous positives were obtained from all environmental water samples. Cells were easily detected and enumerated, ranged in size from 10–35  $\mu$ m, and stained blue-black with Lugol's iodine. A 1-way ANOVA revealed a significant difference between the environmental water sample lipid dose treatments ( $P = 0.001$ ). The 1:100 (v/v) lipid treatment recovered significantly more parasites than the 1:10 (v/v) lipid treatment ( $141 \pm 19$  cells  $L^{-1}$  versus  $3 \pm 2$  cells  $L^{-1}$ , Tukey  $P = 0.001$ ), but not significantly more than the no-lipid control ( $89 \pm 34$  cells  $L^{-1}$ , Tukey  $P > 0.05$ ). The control treatment also produced a recovery rate significantly greater than the 1:10 (v/v) lipid treatment (Tukey  $P = 0.003$ ). A contrast comparing the control versus both lipid treatments was significant ( $P = 0.030$ ); however, as a result of the low recovery in the 1:10 treatment the combined lipid effect was to decrease recovery compared with the control. These data indicate that 10% (v/v) lipid additions may inhibit recovery. Variability across the treatments was low compared with Experiments 2 and 3.

#### Experiment 5

##### Comparison of RFTM Filtration and Immunoassay Methods

Prior to this work, the only other attempts to enumerate planktonic stages of *P. marinus* used a polyclonal immunoassay with near genus-level specificity (Roberson & Dungan 1994, Ragone Calvo et al. 2003, but see Bushek et al. 2002). The present experiment was conducted to compare detection of planktonic *P. mari-*

*inus* via the polyclonal immunoassay and the RFTM filtration methods. Paired environmental water samples (500-mL) were collected September 26, 1998 from Oyster Landing, North Inlet, South Carolina using two automated ISCO water samplers. The paired samples were processed using the RFTM filtration and the immunoassay methods. All samples were prefiltered through 25- $\mu$ m nylon mesh. RFTM samples were processed as described for Experiment 2, treatment 1. The immunoassay samples were processed using methods described by Bushek et al. (2002), which is a modification of the protocol described by Dungan and Roberson (1993). Briefly, the samples were concentrated at  $\times 100$  g for 15 min and resuspended in sterile FSW with 10% buffered formalin. Particulates were then captured on a 1.0- $\mu$ m pore size black polycarbonate filter in a Swinnex-13 membrane filter unit, washed three times with phosphate buffered saline (PBS), blocked with a blocking buffer for 30 min and then washed with phosphate buffered saline containing Tween-20 (PBST). The primary antibody (rabbit anti-*P. marinus*) was applied for 30 min then washed with PBST before the secondary antibody (goat antirabbit-FITC) was applied. After 30 min, the sample was washed with PBST, counterstained with Evan blue, washed with PBS, and finally mounted on slides to enumerate parasites via epifluorescence microscopy. Using this procedure, the nucleus of any *Perkinsus* spp. parasites present fluoresced bright green, making them distinguishable from other cells and debris. Counts from both the immunoassay and RFTM assays were standardized to parasite cells per liter and results were compared using a paired *t*-test.

The RFTM and immunoassay methodologies for enumeration of *Perkinsus* spp. parasites were not significantly different (Paired *t*-test for means  $P = 0.411$ , Table 3). This indicated that both methods were equally sensitive.

#### DISCUSSION

In contrast to Yarnall et al. (2000), we successfully detected and enumerated *P. marinus* in samples collected from enzootic waters using standard RFTM methods. Yarnall (1998) reveals that Yarnall et al. (2000) used centrifugation to concentrate their samples in which their PCR assays did not yield enough DNA for quantitation. Interestingly, a nearby contemporaneous study using a flow cytometric immunoassay estimated planktonic *P. marinus* concentrations of 200–400 parasites  $L^{-1}$  (Ragone Calvo et al. 2003). Our comparison of detection via RFTM versus the immunoassay indicated that both methods were equally sensitive when applied to environmental water samples. In this study, centrifuga-

TABLE 3.

Comparison of RFTM filtration method and immunoassay method. Each replicate represents paired water samples taken at the same time with automated ISCO water samplers. The number of *Perkinsus* spp. parasites detected is reported for each method. The methods were not significantly different ( $\alpha = 0.05$ ).

Replicate	RFTM	Immunoassay
1	13	20
2	33	30
3	23	10
4	7	0
Mean $\pm$ (SEM)	19 $\pm$ (5.71)	15 $\pm$ (6.45)

tion was less efficient than filtration, which may account for the lack of detection by Yarnall et al. (2000) when results from Ragone Calvo et al. (2003) indicated that the parasite was abundant. A more recent study using improved molecular genetic tools was able to quantify *P. marinus* with PCR methods from the same area as the Yarnall et al. (2000) study (Audemard et al. 2004).

It should be noted that the RFTM and immunoassay methods are genus-specific and may detect other species if present. Hence, as species-specific molecular methods become more widely available (e.g., Audemard et al. 2004) they may be necessary where multiple species occur. It may also be valuable to compare species-specific and genus-specific methods in samples collected over space and time. Such experiments will provide insight into the inter and intragenus cross-reactivity of the methods as well as the co-occurrence of *Perkinsus* species.

The low recovery of *in vitro* cultured parasites in this study was disconcerting, but may not be relevant to enumerating *Perkinsus* spp. abundances from environmental samples. Compared with parasites obtained from infected hosts, it was clear that cultured cells did not enlarge or stain well after incubation in RFTM. This difference indicates that *in vitro* cultured *P. marinus* cells are physiologically different from cells harvested from infected hosts. Bushek et al. (1997) found that *in vitro* cultured cells appeared to have a reduced ability to survive injection into oysters, and Ford et al. (2002) clearly demonstrated a reduction in virulence following *in vitro* culture using the same culture methods used in the present study. Our attempts to identify steps in the methodology that account for cell loss were largely unsuccessful, leading us to the conclusion that cell loss is largely a result of handling the cells, possibly from the shock experienced by cells when transferred from culture medium to FSW. This may partly explain the loss of virulence reported by Ford et al. (2002), as they similarly transferred parasites from culture medium to seawater and then into hosts. Interestingly, enlargement, staining and recovery of cultured *P. marinus* improved with the addition of lipids (1:100 v/v); albeit not always significantly, indicating potential deficiencies in the culture medium. Regardless, the RFTM assay and an independent polyclonal immunoassay performed similarly with environmental samples indicating approximately equal detection efficiencies.

Based on the earlier results and discussion, the RFTM filtration method is a simple, inexpensive method for enumerating *Perkinsus* spp. parasites in environmental water samples. Several additional refinements of the method have resulted in a simpler and less time-consuming RFTM filtration method (Fig. 4). Specifically, washing and resuspension of the pelleted, NaOH-hydrolyzed samples in deionized water rather than FSW was convenient, efficient and did not affect sample storage (Gauthier & Fisher 1990). In addition, stained samples may be read on a Hausser counting chamber at 40 $\times$  magnification instead of 100 $\times$  magnification because the natural cells enlarge more during RFTM incubation than *in vitro* cultured cells, stain characteristically and are easily observed under 40 $\times$  magnification. This dramatically decreased the time required to scan the counting chamber. Lipids were not added to the final protocol because it was not clear from the results of Experiments 3 and 4 that lipids actually improved the overall recovery of planktonic parasites because of the high variability and reduced recovery in 1:10 (v/v) lipids/RFTM samples.

Direct water borne transmission of *P. marinus* was discovered more than 50 y ago (Ray 1954), yet little is known about the free-living stages of this or any other *Perkinsus* species that afflict various molluscs around the globe. As these protozoan pathogens

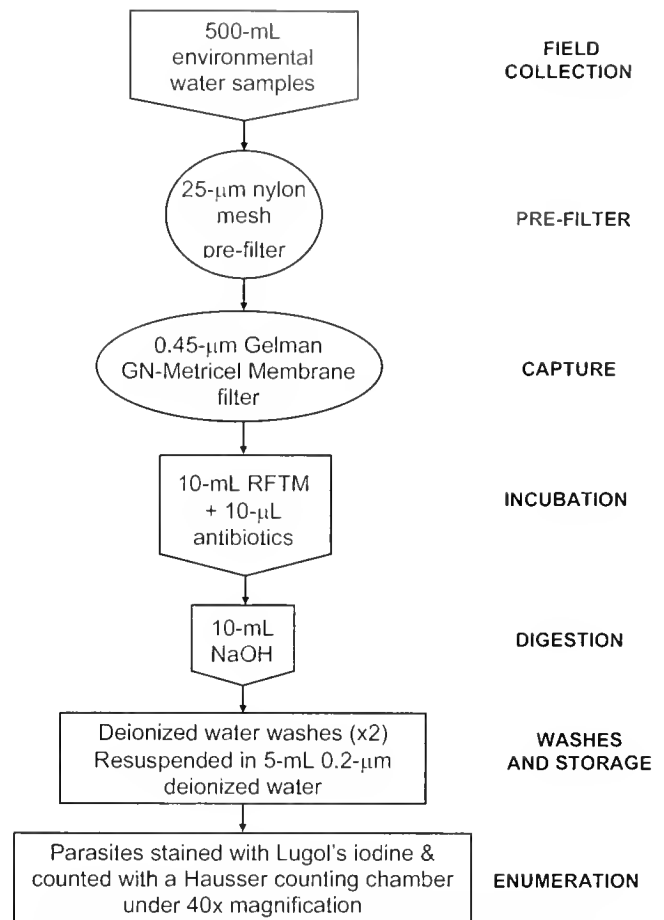


Figure 4. Flow chart of the adopted RFTM filtration method.

continue to plague shellfisheries, the need to better understand their dispersal and transmission dynamics continues to grow. A thorough understanding of these dynamics can be achieved only through investigations exploring the timing and fate of free-living planktonic stages during transmission. The experiments discussed in this study outline a simple method to detect and enumerate planktonic stages of *Perkinsus* spp. The method adapts the well-known and extensively applied RFTM method that is known to detect all species of the genus *Perkinsus*. Hence, the method should be readily accessible to most laboratories that already use RFTM assays. Whereas the RFTM filtration method provides a simple and inexpensive methodology for enumerating *P. marinus* in environmental water samples, the absolute efficiency of the method remains questionable. Nonetheless, the RFTM filtration method may be used to indicate relative differences in parasite numbers.

#### ACKNOWLEDGMENTS

The authors thank K Hudson for assistance with culturing *P. marinus* isolates. M Chintala and J LaPeyre provided some of the isolates and C Dungan provided several discerning comments in developing these experiments. Funding for this research was provided by NSF-RUI Project #DEB-950957, and South Carolina Sea Grant Consortium ODRP Project R/OD-1.

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## COMPARISON OF TECHNIQUES FOR DIAGNOSIS OF BROWN RING DISEASE AND DETECTION OF *VIBRIO TAPETIS* IN THE MANILA CLAM, *VENERUPIS (RUDITAPES) PHILIPPINARUM*

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**ABSTRACT** The bacterium, *Vibrio tapetis*, is the aetiological agent of Brown Ring Disease (BRD), which affects the Manila clam, *Venerupis (Ruditapes) philippinarum*. Two PCR assays for detection of *V. tapetis* were applied to a sample of 52 Manila clams, and evaluated in comparison with the more traditional *V. tapetis* detection method of microbiological isolation and characterization, as well as the traditional BRD diagnostic technique of shell valve analysis. The pathogen was detected in 15.4% of the sample using the PCR assay of Rodriguez et al. (2003, 2006) in 50% of the sample using the PCR assay of Paillard et al. (2006) and in 36.5% of the sample by microbiological methods. Whereas shell valve analysis was the least sensitive technique, detecting BRD in 7.7% of the sample, it was an essential diagnostic tool because it was the only technique that identified the disease, rather than the aetiological agent. None of the four techniques was sufficient on its own for effective BRD diagnosis; rather various combinations of two techniques were the minimum required. The combination of shell valve analysis with the assay of Paillard et al. (2006) proved to be the most sensitive and rapid of those tested. Shell valve analysis was the most time-efficient and cost-effective technique, whereas microbiological characterization was the most time-consuming, and PCR detection, using either set of primers, the most expensive. These results may need to be considered in light of diagnosis and management of BRD.

**KEY WORDS:** brown ring disease, *Vibrio tapetis*, *Venerupis (Ruditapes) philippinarum*, diagnostics, PCR, microbiology

### INTRODUCTION

The Manila clam, *Venerupis (Ruditapes) philippinarum* (Adams & Reeve, 1850), is the second most important cultivated mollusc species in the world, contributing 20% of the total world aquaculture production of molluscs (FAO 2002). Globally, production of the Manila clam has increased by 60% from 1997 to 2002 (FAO 2002). In Europe, production takes place predominantly in Italy, France, Spain and the United Kingdom. Ireland's Manila clam production, though small, has been steadily increasing in recent years, with an output of 181 tons achieved in 2004 (Parsons 2005). The increase in production worldwide, with its concentration of large quantities of clams in confined areas, has facilitated the proliferation of pathogens such as *Perkinsus* sp. (in Asia) (Park & Choi 2001, Park et al. 2006a) and *Vibrio tapetis* (in Europe and Asia) (Paillard 2004, Park et al. 2006b). Consequently, there is a need for rapid, effective and accurate techniques for the diagnosis of Manila clam diseases.

Brown ring disease (BRD) caused by the bacterial pathogen, *V. tapetis*, is not regulated for disease control under existing European Union (EU) legislation, nor is it a disease notifiable to the Office International des Epizooties (OIE). Nevertheless, since the early 1990s, it has been responsible for mass mortalities in the clam cultivation industry in Europe, and has been diagnosed from the majority of clam cultivation areas (Paillard & Maes 1990, Castro et al. 1992, Paillard et al. 1994, Robledo et al. 1994, Castro et al. 1995, Figueras et al. 1996, Castro et al. 1997, Novoa et al. 1998, Clarke 1999, Allam et al. 2000b).

Diagnostic methods for BRD have traditionally involved examination of the interior shell valves for analysis of conchiolin deposition, as described by Paillard & Maes (1994). Histopathology is not useful for BRD diagnosis because tissue lesions are not systematically observed in diseased clams (Paillard et al. 1994) and alterations of the digestive gland and mantle are observed only

in the more advanced stages of the disease (Plana & Le Pennec 1991, Paillard et al. 1994, Paillard 2004). Notwithstanding the analysis of shell valves for BRD signs, all other diagnostic techniques focus on detection of the aetiological agent, *V. tapetis*, rather than the disease itself. Bacteriological isolation procedures are in place and have resulted in the isolation of *V. tapetis* from BRD-affected clams from France, England and Galicia, northwestern Spain (Paillard & Maes 1990, Borrego et al. 1996, Novoa et al. 1998, Allam et al. 2000b). An immunological assay was developed in southwestern Spain, which demonstrated that *V. tapetis* was the aetiological agent of BRD in this region (Castro et al. 1997). An enzyme-linked immunoassay (ELISA), using monoclonal antibodies, which react specifically with *V. tapetis*, is also available for identification of the bacterium (Noel et al. 1996).

Molecular techniques are now available for detection of *V. tapetis*, in the form of two polymerase chain reaction (PCR) assays, each of which amplifies a specific target region of *V. tapetis* DNA (Rodríguez et al. 2003, 2006, Paillard et al. 2006). Previous studies on other pathogens in the same bivalves have indicated that PCR amplification has the ability to detect a higher prevalence of a pathogen than traditional diagnostic methods (Carnegie et al. 2000, Harwood et al. 2004, Lynch et al. 2006).

The aims of this study were to apply *V. tapetis*-specific PCR assays for *V. tapetis* detection and to evaluate them against the more traditional detection method of microbiological isolation and characterization, as well as the traditional BRD diagnostic technique of shell valve analysis; and to assess which technique, or combination of techniques, is most suitable for BRD diagnosis and *V. tapetis* detection in terms of efficiency of detection, time required and cost-effectiveness.

### MATERIALS AND METHODS

#### Clams

A sample of 52 clams, of 25–30 mm in length, was obtained from Mulroy Bay, Donegal, northwest Ireland, in April 2005, from

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a cultured stock in which BRD was suspected. Clams were placed in a cold room at 4°C prior to dissection, to encourage relaxation of their adductor muscles, for easier opening of the shell valves. After opening of the valves, over 200 µL of extrapallial fluid (EPF) was extracted from each individual by inserting a needle between the mantle and interior shell valve. The EPF was placed in a labeled eppendorf tube. The soft parts of the animal were discarded. The shell valves were retained, labeled and left to dry for 24 h.

#### Macroscopic and Microscopic Analysis of BRD on Shell Valves

The stage of progression of BRD on the shell valves was monitored according to the classification system of Paillard & Maes (1994), in which the syndrome is characterized by two stages, which may occur simultaneously in a given individual: a conchiolin deposit stage (CDS), in which layers of an organic conchiolin deposit are laid down between the mantle edge and periostracal lamina, and a shell repair stage (SRS), in which the clam lays down layers of shell on top of the conchiolin deposit, for repair and recovery from the disease. CDS index is based on the use of a scale, which takes into account the extent and thickness of the brown ring deposit. For example, CDS 1 (Stage 1) is not visible to the naked eye and can be seen only with a microscope. CDS 2 (Stage 2) corresponds to a limited deposit and CDS 3 through to CDS 7 to more extensive deposits on the inner shell surfaces. Three stages of shell repair are defined: SRS 1 corresponds to the initial accumulation of calcified material; SRS 2 to the partial covering of the deposit by wide calcified plates and SRS 3 to the completely covering of the deposit by shell layers. When the processes of conchiolin deposition (CDS) and shell repair (SRS) are considered together, three phases of the disease are defined: Phase 1 corresponds to development of the disease and is characterized only by accumulation of conchiolin; Phase 2 is characterized by the simultaneous occurrence of both processes in a given individual; and Phase 3 corresponds to the end of the recovery process, when the individual has completely covered the conchiolin deposit (Paillard & Maes 1994).

#### Microbiological Isolation and Characterization of *Vibrio tapetis*

One-hundred micro liters of EPF from each individual was spread, using a "hockey stick" spreader, on a Thiosulfate Citrate Bile Sucrose (TCBS) (Oxoid Ltd.) agar plate and incubated for 24 h at 18°C. TCBS agar is selective for *Vibrio* species: *V. tapetis* is saccharose-negative on TCBS agar and, as such, appears as a green colony. Four green, saccharose-negative colonies from each TCBS plate were selected and isolated on marine agar to obtain pure cultures. They were subsequently stored in vials of 15% glycerol/85% marine broth at -80°C until further characterization. All the remaining colonies were collected from each TCBS plate, placed in eppendorf tubes containing 1 mL sterile distilled water (in order that each tube contained the colonies of an individual clam) and stored at -20°C until further analysis by PCR.

Isolated colonies were characterized by a series of standard morphological, physiological and biochemical tests (Cowan 1974, Smibert & Krieg 1981, West & Colwell 1984, Borrego et al. 1996). All biochemical tests were performed using marine agar (DIFCO) unless otherwise stated and the incubation temperature was 18 ± 1°C unless otherwise stated. The tests, and the criteria for characterization as *V. tapetis*, are listed in Table 1. The ingredients and recipes used in each test are described in Appendix 3. The type *V. tapetis* strain, CECT 4600 was used as a positive control for all

TABLE 1.  
Characterization tests for *Vibrio tapetis* isolates

Growth in:	
0.0% NaCl	Negative
1.5% NaCl	Positive
2.0% NaCl	Positive
6.0%	Negative
Growth at:	
4°C	Positive
22°C	Positive
30°C	Negative
Gram Reaction	Negative
Motility	Positive
Morphology	Curved Rods
Oxidase	Positive
Oxidation/Fermentation	Fermentative
Catalase	Positive
Susceptibility to O/129 (150µg)	Susceptible
Amino Acids:	
Arginine Dihydrolase	Negative
Lysine Decarboxylase	Negative
Ornithine	Negative
Decarboxylase	
Amylase	Positive
Gelatine Liquefaction	Positive
Lipase	Positive
Esculine	Positive/Negative
Nitrate Reduction	Positive
Indole	Positive
Gas from Glucose	Negative

tests. Isolates, which did not meet the *V. tapetis* criteria, were eliminated from further tests as results became available. Colonies that met the requirements for characterization as *V. tapetis*, had their identities confirmed by PCR detection of *V. tapetis* DNA (Rodríguez et al. 2003, 2006).

#### PCR Detection of *Vibrio tapetis*

Two PCR assays were used for the detection of *V. tapetis*: the first, developed by Rodríguez et al. (2003, 2006), uses template DNA extracted from bacterial colonies, and the second, developed by Paillard et al. (2006), employs template DNA extracted from the EPF of clams.

#### Rodríguez Assay (2003, 2006)

Colonies from TCBS plates, in suspension in sterile distilled water, were thawed. DNA from individual clams was extracted using InstaGene Matrix (BIO-RAD) according to manufacturers' instructions and stored at -20°C until required for PCR reactions. PCR amplification of template DNA (at a concentration of 1-3 µg) was undertaken using one Pure Taq Ready-To-Go PCR Bead (Amersham Biosciences) in every reaction: each bead contained 1.5 units of Taq polymerase, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1 mM MgCl<sub>2</sub> and 200 mM each dNTP. For a 25-µL reaction, the bead was combined with 2 pmol (1 µL) of forward primer, 2 pmol (1 µL) of reverse primer, 22 µL of sterile distilled water and 1 µL of DNA template solution. DNA from *V. tapetis* CECT 4600 was used as a positive control and distilled deionized water was used as a negative control. Amplification was performed in a Hybaid thermal cycler programmed as follows: an initial denaturation step at

94°C for 3 min; 35 cycles of 94°C for 60 sec, 64°C for 60 sec and 72°C for 90 sec; and a final cycle of 72°C for 5 min. The PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide and photographed under UV light. A 50–2,000 bp ladder (Sigma Chemical Company) was used as a molecular marker. In cases where either the positive or negative control was not confirmed by PCR as positive and negative respectively, the results were discarded and the analysis repeated. PCR results were graded as either negative or positive.

#### Paillard Assay (2006)

DNA was extracted from 100 µL of EPF, of each of the 52 clams, using the chelex method (Saulnier et al. 2000). PCR reactions were carried out in a total volume of 50 µL, including 10 mM Tris-Cl, 50 mM KCl containing 200 µM of each dNTP, 1.5 mM MgCl<sub>2</sub>, 400 µM of each primer, VtF and VtR, 0.2U of Taq DNA polymerase (Sigma) and 200 ng of genomic DNA (5 µL of template DNA). DNA from *V. tapetis* CECT 4600 was used as a positive control and distilled deionized water was used as a negative control. PCR amplification was performed in a Hybaid thermal cycler and the thermal cycle consisted of an initial denaturation step at 94°C for 3 min, followed by 35 cycles of 94°C for 60 sec, 63°C for 60 sec and 72°C for 45 sec. The PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide and photographed under UV light. A 50–2,000 bp ladder (Sigma Chemical Company) was used as a molecular marker. In cases where either the positive or negative control was not confirmed by PCR as positive and negative respectively, the results were discarded and the analysis repeated. PCR results were graded as either negative or positive.

#### Comparison of Techniques for Diagnosis of BRD and Detection of *Vibrio tapetis*

The results of screening for BRD by shell analysis, *V. tapetis* detection by bacteriological techniques and *V. tapetis* detection by two PCR assays were subsequently compared. A chi-square test was used to ascertain if there were significant differences in the detection rate between each diagnostic technique.

### RESULTS

#### Macroscopic and Microscopic Analysis of BRD on Shell Valves

Prevalence of BRD in the sample of 52 clams was 7.7% as classified by the system of Paillard & Maes (1994); 48 did not

exhibit BRD symptoms. Of the four symptomatic clams, one displayed CDS 2 and no shell repair, and so was in Phase 1 of BRD; two clams exhibited CDS 3 and SRS 1, and thus were in Phase 2 of BRD and the fourth clam showed CDS 5 and SRS 1 and was thus also in Phase 2 of BRD. Two (50%) were BRD/*V. tapetis*-positive by other diagnostic techniques: Table 2 shows that none of the shell analysis positives were detected by microbiology; one was detected by the Rodríguez assay and two were detected by the Paillard assay. There appeared to be no correlation between stage of disease and detection by other techniques; the clam displaying CDS 5 and one of the clams displaying CDS 3 were not detected as positive by any of the other methods; the second clam exhibiting CDS 3 was positive by both PCR assays; whereas the individual showing CDS 2 was positive by the Paillard assay. Two (50%) of the animals positive by shell analysis were not detected as positive by any other technique. Of 48 animals negative by shell analysis, 31 (64.6%) were deemed positive by one or more of the other techniques.

#### Microbiological Isolation and Characterization of *Vibrio tapetis*

From 52 TCBS plates, each plate representing an individual clam, a total of 208 suspected *Vibrio* colonies were isolated. After salt and temperature tests (Table 1), 162 colonies were eliminated. Following characterization by the remaining tests, a total of 23 isolates from 19 individuals were identified as *V. tapetis*. Sixteen of the 23 (70%) isolates were derived from individual clams, whereas two isolates originated from each of two clams, and three isolates originated from one individual. Overall, *V. tapetis* was detected in 19 individuals (36.5%) of the sample, 13 (68%) of which were also detected by at least one other technique. Table 2 shows that none of the microbiology *V. tapetis*-positive individuals was positive by BRD shell analysis, three were positive by the Rodríguez assay and 13 by the Paillard assay. Six (32%) of the microbiology positives were not detected by any of the other techniques and 17 (52%) microbiology negatives were deemed positive by one or more techniques.

#### PCR Detection of *Vibrio tapetis*

##### Rodríguez Assay (2003; 2006)

*V. tapetis* was detected in 15.4% (8 individuals) of the sample. Six (75%) of these positives were also positive by other diagnostic methods, of which, one was detected by BRD shell analysis; three by microbiology; and six by the Paillard assay (Table 2). Twenty-

TABLE 2.

Comparison of positive and negative results between four techniques for diagnosis of Brown Ring Disease/detection of *Vibrio tapetis* in a sample of Manila clams, *Venerupis (Ruditapes) philippinarum*. The first column shows the numbers of positives and negatives detected by each individual technique and the following columns show the comparative numbers of positives and negatives detected by each of the remaining techniques. *n* = 52.

Positives and Negatives Detected by Each Technique	Shell Analysis +	Shell Analysis –	Microbiology +	Microbiology –	Rodríguez PCR +	Rodríguez PCR –	Paillard PCR +	Paillard PCR –
Paillard PCR + (26)	2	24	13	13	6	20		
Paillard PCR – (26)	2	24	6	20	2	24		
Shell Analysis + (4)			0	4	1	3	2	2
Shell Analysis – (48)			19	29	7	41	24	24
Microbiology + (19)	0	19			3	16	13	6
Microbiology – (33)	4	29			5	28	13	20
Rodríguez PCR + (8)	1	7	3	5			6	2
Rodríguez PCR – (44)	3	41	16	28			20	24

six (59%) of the negatives by the Rodríguez assay were not confirmed by any of the other techniques.

#### Paillard Assay (2006)

*V. tapetis* was detected in 50% (26 individuals) of the sample. Seventeen (65%) of the 26 positives were confirmed by other diagnostic methods, of which 2 were detected by BRD shell analysis; 13 by microbiology and 6 by the Rodríguez assay (Table 2). Nine (35%) Paillard PCR-positives were not detected by any other technique and 10 (39%), which were negative by the Paillard assay were detected as positive by one or more of the other techniques.

#### Comparison of Techniques for Diagnosis of BRD and Detection of *Vibrio tapetis*

The four techniques detected BRD and *V. tapetis* at different levels within the same sample (Table 3) and there was a significant difference between the numbers of BRD/*V. tapetis* positives and negatives detected by the four methods ( $\chi^2_3 = 29.45$ ,  $P < 0.001$ ). BRD prevalence detected by shell analysis was 7.7%; *V. tapetis* detection by isolation and characterization was 36.5%; and by the primers of Rodríguez et al. (2003, 2006) and Paillard et al. (2006) was 15.4% and 50% respectively. When two diagnostic techniques were applied, detection levels ranging from 21.2% to 61.5% were revealed: a combination of BRD shell analysis and PCR by Rodríguez et al. (2003; 2006) produced the lowest detection levels at 21.2%, whereas the combination of microbiological characterization and PCR by Paillard et al. (2006) produced the highest detection levels at 61.5%. Applications of three techniques led to detection levels ranging from 51.9% to 65.4%, and finally, a combination of all four techniques detected the highest levels of BRD/*V. tapetis* (i.e., 69.2%).

Shell valve analysis was found to be the most cost-efficient technique; only a microscope was required. Microbiological meth-

ods entailed the cost of a wide variety of reagents and media; but the most expensive techniques were *V. tapetis* detection by PCR. With regards to time-efficiency, BRD diagnosis by shell analysis of 52 clams was completed in less than two hours. Only four individuals were BRD-positive and the time taken would be expected to be longer in samples containing a higher percentage of diseased individuals, because of the time required to establish the CDS and SRS of each individual. PCR detection by the Paillard et al. (2006) primer was the next most time-efficient method, with the whole process from DNA extraction through to the visualization of PCR products on an electrophoretic gel taking less than 10 h. The Rodríguez et al. (2003; 2006) assay entailed growth of bacterial colonies on TCBS agar prior to DNA extraction and this added a further 24–48 h to the process. By far the most time-consuming technique was the bacteriological method, which required much preparatory and manipulative work. Additionally, *V. tapetis* colonies require approximately 24–48 h growth on agar media for isolation and the same time or longer for the characterization tests that follow. In this study, the procedure from colony isolation from the extrapallial fluid through to characterization as *V. tapetis* took 5–6 days to complete for any one bacterial isolate.

#### DISCUSSION

Developments in molecular biology have led to new methods for diagnosing shellfish diseases. The advantages of molecular techniques such as PCR are that they are potentially faster and more sensitive than methods such as culture, serology and histology that are traditionally used to identify shellfish diseases (Cunningham 2002). The currently available PCR primers for detection of *V. tapetis* in clam tissue differed significantly in detection rate with the primers by Rodríguez et al. (2003, 2006) detecting fewer positives (15.4%) than those by Paillard et al. (2006) (50%). Optimization of reaction conditions for the Rodríguez primers was required prior to use, but not for the Paillard primers. However, this optimization may have been necessary because of geographic variation in *V. tapetis* strain (Romalde et al. 2002, Paillard et al. 2006) and perhaps the strain detected in Mulroy Bay was more amenable to detection by the Paillard assay than by the Rodríguez assay. The two sets of primers also differed with respect to their source DNA material: the Paillard primers were applied to the clam's EPF, whereas the Rodríguez primers were applied to DNA from bacterial colonies. Culture of *V. tapetis* is often met with variable success, depending on the stability of culture conditions and the composition of the microbiota and could possibly have compounded the lower detection rate of the Rodríguez assay. Overall, *V. tapetis* detection by PCR was the fastest technique used in the current study, thus making it suitable for situations in which a rapid diagnosis is required. Because PCR is used to amplify certain regions of DNA, it can also achieve significant increases in the sensitivity of detection (McPherson et al. 1991). This was demonstrated by the Paillard assay, which was the single most sensitive assay, capable of detecting *V. tapetis* levels down to  $10^2$  cfu mL<sup>-1</sup> (Paillard et al. 2006).

In this study, nine PCR-positives by the Paillard assay and two by the Rodríguez assay went undetected by any one of the other techniques and could potentially have signified false positives. One of the limitations of PCR is that when used in disease diagnosis, false positives identifying DNA similar to, but not of, the target organism can occur, because of low specificity of the primers. To ensure the greatest possible specificity of PCR primers to

TABLE 3.

A comparison of the efficiency of detection of BRD/*Vibrio tapetis* in a sample of 52 *Venerupis (Ruditapes) philippinarum* by different techniques, individually and in combination.

Technique/Combination of Techniques	Percentage Detection
BRD shell analysis	7.7
Microbiological characterization	36.5
Rodríguez assay	15.4
Paillard assay	50
BRD shell analysis + Microbiological characterization	44.2
BRD shell analysis + Rodríguez assay	21.2
BRD shell analysis + Paillard assay	53.8
Microbiological characterization + Rodríguez assay	46.1
Microbiological characterisation + Paillard assay	61.5
Rodríguez assay + Paillard assay	53.8
BRD shell analysis + Microbiological characterization + Rodríguez assay	51.9
BRD shell analysis + Microbiological characterization + Paillard assay	65.4
BRD shell analysis + Rodríguez assay + Paillard assay	57.7
Microbiological characterization + Rodríguez assay + Paillard assay	65.4
BRD shell analysis + Microbiological characterization + Rodríguez assay + Paillard assay	69.2



the target DNA, DNA sequencing of PCR positives prior to routine use of the primers is recommended (Cunningham 2002). In the current study, PCR products obtained using the Paillard et al. (2006) primers were sequenced and found to be consistent with reference *V. tapetis* sequences from the European Molecular Biology Laboratory (EMBL) database, thereby suggesting that the nine PCR positives, detected by this assay only, reflected real *V. tapetis* DNA. In general, however, a PCR positive result alone is not considered enough to confirm the presence of a pathogen, and should be supported by other diagnostic methods before a confirmatory diagnosis is obtained (Walker & Subasinghe 2000).

Two individuals, who displayed brown conchiolin deposits, went undetected for *V. tapetis* by both PCR assays. It is possible that the conchiolin was deposited in response to an agent other than *V. tapetis*, because conchiolin deposition unassociated with *V. tapetis* has been observed in other bivalves (Sindermann 1990, Paillard 2004); this seems unlikely, however, as the pathogen was detected in other individuals within the current sample. Alternatively, the deposits may have persisted in these animals from the conchiolin deposit stage (developmental) of the disease, but the causative agent may have been reduced to nondetectable levels during the recovery stage: the shell repair process is associated with a decrease in *V. tapetis* burden (Allam et al. 1996). The PCR-negative results could also be explained by the pathogen being localized predominantly in tissues other than those targeted for DNA extraction and PCR. In this case, PCR analysis was carried out on the EPF of individuals, but *V. tapetis* is also known to heavily colonize the periostracal lamina and the mantle surface (Paillard & Maes 1995a, 1995b; Allam et al. 1996, Paillard 2004). It is possible that in these two individuals, the pathogen was localized predominantly in the periostracal lamina or on the mantle surface, neither of which was analyzed by PCR and therefore went undetected by both assays.

Isolation and characterization of *V. tapetis* by bacteriological methods produced detection levels of 36.5%, the second highest detection level of the single techniques. However, the lack of sensitivity of the technique is highlighted by the fact that a significant proportion of its negatives (17 of 33) were detected as positive by one or more of the other techniques. Problems associated with the microbiological isolation and characterization of *V. tapetis* have been described in previous studies. In southwestern Spain, microbiological methods failed to detect *V. tapetis* in cultured Manila clams displaying disease signs identical to those of BRD (Castro et al. 1992, 1995; 1997). In bacteriological analyses of the clams' conchiolin deposits, none of the isolates were phenotypically typed as *V. tapetis* (Castro et al. 1997). The pathogen was subsequently detected by an indirect immunofluorescence technique and the authors suggested that lower pathogen levels, detected in *V. philippinarum* from this region in comparison with BRD-affected clams from other regions, were the reason that standard microbiological methods failed to isolate the pathogen (Castro et al. 1997). Similarly, Paillard (2004) stated that isolation of *V. tapetis* might not provide a clear diagnosis because the bacterium grows slowly and is generally nonpredominant within the total heterotrophic microflora. Immunological techniques for the detection of bacterial pathogens in other species have previously been found to be more sensitive than classical microbiological tests (Bernoth 1991, Lee & Gordon 1987).

Nevertheless, isolation of the causative agent is often essential in diseases of a bacterial etiology. Microbiological detection proved to be the most time-consuming and labor-intensive of the

techniques in the present study. In epidemiological studies, involving large sample sizes of clams, the time taken and labor required for completion of microbiological tests would render this technique unsuitable. There are commercial kits available, such as the API 20E (BioMérieux, France), for the rapid identification of *Vibrios*, but whereas previous studies have found them to be effective for *V. tapetis* identification (Allam et al. 2000b, Jensen et al. 2003), the authors of the current study found them unreliable (unpubl. data).

BRD diagnosis has traditionally been achieved by analysis of the brown conchiolin deposit on the shell valves (Paillard & Maes 1994, Allam et al. 1996, Figueras et al. 1996, Paillard et al. 1997, Novoa et al. 1998, Allam et al. 2000a, 2000b; 2001, Reid et al. 2003b, Paillard et al. 2004, Soudant et al. 2004). Whereas it proved to be the least sensitive technique in the current study, failing to detect many positives detected by the other techniques, it is essential for BRD diagnosis: it is the only technique of those used in this study, which detected the actual disease, as opposed to the aetiological agent, which is detected by PCR detection and microbiological isolation. Shell analysis is also useful for classification of BRD into its stages, and thus its assessment of how chronic the infection is and the state of recovery in an individual (Paillard & Maes 1994).

In the current sample, just four animals were BRD-positive as detected by shell analysis, yet up to 32 animals were positive for *V. tapetis* (by a combination of the other techniques). This suggests that there was far more *V. tapetis* than BRD in the sample, and that this may also be true of *V. tapetis* and BRD in the environment. The bacterium has already been detected in other clam species (e.g., *R. decussatus* and *Venerupis aurea*; Maes & Paillard 1992, Novoa et al. 1998) and in other animals such as the cockle, *Cerastoderma edule* (Maes & Paillard 1992), the wrasse *Symphodus melops* (Jensen et al. 2003) and the halibut, *Hippoglossus hippoglossus* (Reid et al. 2003a), suggesting it is widespread in the environment in general. Like most *Vibrios*, it is likely to be an opportunistic pathogen, which does not always generate disease: *V. philippinarum* has been shown to harbor *V. tapetis* without experiencing BRD and associated mortalities (unpubl. data; Castro et al. 1997). Consequently, the detection of *V. tapetis* alone is not sufficient for a clear diagnosis of BRD, because it is not indicative of disease. The observation of clinical disease signs on the hosts' shells, even if only in small numbers as in the current sample, is necessary. Shell valve analysis is thus an essential tool in BRD diagnosis. An exception perhaps arises in cases of acute disease, in which the clam dies prior to manifestation of brown ring signs: this has occurred after injection of *V. tapetis* into the clam's extrapallial space or adductor muscle (Allam et al. 2002). There is no known documented evidence of such an occurrence in the wild.

The major drawback of BRD diagnosis by shell analysis alone is that the brown ring symptom is neither exclusive to BRD, nor exclusive to clams. Essentially, the brown conchiolin deposit is a defense mechanism and has been observed in many bivalves undergoing infection such as oysters (Perkins 1996, Cuif & Dauphin 1996) and abalone (Sherperd & Huchette 1997). See review by Paillard (2004). Parasites such as fungi, annelids and trematodes can disturb the extrapallial area by boring into or irritating the mantle epithelium (Sindermann 1990, Paillard et al. 1994). As such, brown rings on shell valves of clams are not exclusively caused by infection by *V. tapetis*. For this reason, a diagnosis of BRD based on shell analysis alone is insufficient; evidence of the aetiological agent is also required.

A combination of diagnostic techniques is therefore necessary for an accurate BRD diagnosis. The results here demonstrated the effectiveness of various combinations. The more techniques used in a particular combination did not necessarily lead to a higher detection rate, although the use of all four methods produced the highest detection levels. There was significant variability in detection rates when various combinations of two techniques were used, from 21.2% to 61.5% detection in the sample; each combination of two tests involving the Paillard assay produced detection levels above 50%, whereas those not using the assay produced detection levels below 50%. It would appear therefore that for sensitive detection, any combination of two techniques should involve the assay of Paillard et al. (2006). There was less variability in detection levels when combinations of three techniques were used: 51.9% to 65.4%. Diagnosis by a combination of three techniques provided detection levels not dissimilar to those produced from a combination of all techniques (i.e., 69.2%), indicating that it is unnecessary to use all four diagnostic methods in an analysis, unless the objective is to ensure, as far as possible, that clams are BRD/*V. tapetis*-negative.

In BRD management, the diagnostic methods, or combinations thereof, applied in an analysis of clams for BRD/*V. tapetis* will depend on a number of factors, specifically, the information required from the analysis; the number of individual clams in the sample to be analyzed; the time available and the financial resources available. A clam sample should first be assessed for overt BRD signs on the shell and then for the presence of the aetiological agent. The testing of clam seed for *V. tapetis*/BRD in the hatchery prior to purchase would best be accomplished by analyses for detection of the pathogen, as clinical BRD signs cannot be observed in juveniles under 2 mm (and in larvae) (Paillard et al. 2006).

In conclusion, the analyses of diagnostic techniques carried out in the current study demonstrate that none of the four techniques

was sufficient on its own for an accurate diagnosis of BRD. BRD shell analysis was an essential technique and is recommended as the first technique in the analysis of a clam sample for BRD, followed by confirmation, either by PCR or microbiology, that the brown deposits are caused by *V. tapetis*. The combination of BRD shell analysis and PCR detection according to Paillard et al. (2006) was the most sensitive and rapid combination in this study. In instances where isolation of the aetiological agent is required and time is not a limiting factor, BRD shell analysis in conjunction with microbiological techniques is recommended. PCR detection is valuable if sensitivity for detection of lower pathogen levels is required. A comparison of the cost-efficiency of each method favored BRD shell analysis, followed in increasing cost by bacteriological isolation and characterization, and finally, either of the two sets of primers.

The results here were based on a single sample, and the sample size of 52 individuals is less than the 60 recommended by Simon & Schill (1984) for detection of an infection present at 5% prevalence in a population of  $1 \times 10^6$ . Additionally, the sample represents a single month, and therefore does not consider seasonal variation in BRD and *V. tapetis* prevalence. Further studies, which would encompass seasonal sampling, are required for validation of the detection efficiency of each technique. However, the results here provide a basis for deciding on diagnostic and detection techniques of choice for BRD and *V. tapetis* in different situations.

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## FLUOROMETRIC MEASUREMENT OF OXIDATIVE BURST IN LOBSTER HEMOCYTES AND INHIBITING EFFECT OF PATHOGENIC BACTERIA AND HYPOXIA

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**ABSTRACT** The stimulation of hemocytes during phagocytosis leads to the generation of a series of oxygen radicals known as reactive oxygen species (ROS). Among these, hydrogen peroxide plays an important microbicidal role by directly killing microorganisms or by serving as an intermediate for other antimicrobial radicals. In this study, we adapted a technique using 2',7'-dichlorofluorescein-diacetate (DCFH-DA) to measure  $H_2O_2$  production in lobster hemocytes. After oxidation by hydrogen peroxide, this molecule produces a fluorescent product that can be easily detected. The respiratory burst was successfully activated in lobster hemocytes by the addition of zymosan particles, but not with phorbol myristate acetate. After optimization, we used the technique to investigate the effect of different bacterial strains, including lobster pathogens, on the oxidative burst. Results demonstrate that *Aureococcus viridans*, a bacterial pathogen that is able to survive phagocytosis by lobster hemocytes, quenches ROS production. The comparison of ROS production in lobsters collected from field sites submitted to different levels of dissolved oxygen suggests that this technique provides a good indicator of lobster physiological status and immunocompetency.

**KEY WORDS:** lobster, *Homarus americanus*, oxygen, *in vitro*, dichlorofluorescein diacetate, ROS

### INTRODUCTION

The American lobster, *Homarus americanus*, is one of the most commercially important species in the Northeastern United States and Canada. This species is subject to different infections, including those caused by bacterial agents such as the Gram-positive *Aerococcus viridans* (Stewart et al. 1969, Battison et al. 2003) and the Gram-negative *Vibrio fluvialis* (Tall et al. 2003), as well as those related to protozoan parasites such as *Paramoeba* (Mullen et al. 2004) and the ciliate *Anophryoides hemophila* (Cawthorn 1997, Athanassopoulou et al. 2004). Against these pathogens, lobsters have a set of cellular and humoral defense factors. In common with other crustaceans, host defense in lobsters is nonspecific, based on activities of circulating hemocytes (Bauchau 1981, Paterson & Stewart 1974, Paterson et al. 1976, Cornick & Stewart 1978, Battison et al. 2003). In these animals, hemocytes defense functions include coagulation, phagocytosis, encapsulation and wound repair (Bauchau 1981, Johansson & Söderhäll 1989, Bachère et al. 1995) and synthesis and secretion of humoral defense factors (Destoumieux et al. 2000, Bachère et al. 2004).

The stimulation of hemocytes during phagocytosis leads not only to release of lysosomal enzymes, but also to superoxide radical ( $O_2^-$ ) generation catalyzed by NADPH oxidase associated with the cytoplasmic membrane. Superoxide radical is metabolized to hydrogen peroxide ( $H_2O_2$ ) by superoxide dismutase (SOD), and the resulting peroxide is further converted to hypochlorite ( $OCl^-$ ) by myeloperoxidase (MPO). Hydrogen peroxide, superoxide radical and hypochlorous acid exhibit direct antimicrobial activity and are widely called reactive oxygen species (ROS) or intermediates (ROI). This phenomenon, known as respiratory burst, plays an important microbicidal role (Anderson 1996). Prior studies reported that ROS production is inhibited in shellfish experimentally submitted to environmental (pollutants, hypoxia) and pathogenic (bacterial) stresses (Anderson 1994, Anderson et al. 1997, Lambert & Nicolas 1998, Le Moullac et al. 1998, Cheng et al. 2002, Lambert et al. 2001).

Because of their importance in the defense process, various tools have been developed to measure ROS production in verte-

brates, including microscopy, luminescence methods and flow cytometry techniques. In their recent paper, Anderson & Beaven (2005) described a chemoluminescence method measuring the oxidative burst in lobsters after activation with Phorbol myristate acetate (PMA). An appealing alternative to this technique is a method using 2',7'-dichlorofluorescein-diacetate (DCFH-DA), initially developed in human phagocytic cells (Rosenkranz et al. 1992). After oxidation by ROS (particularly  $H_2O_2$ ), this molecule produces a fluorescent product that can be easily detected using fluorescence microscopy, or measured using appropriate fluorometers (plate readers, flow cytometers).

There were several goals for this study. First, a simple fluorometric technique allowing detection and quantification of ROS production in lobster hemocytes was devised and optimized. This technique was then used to investigate the effect of pathogenic (bacterial) challenge on ROS production. Finally, as field evaluation of the optimized method, this study examined ROS production in lobsters collected from three different locations in Long Island Sound known to be submitted to different levels of dissolved oxygen.

### MATERIALS AND METHODS

#### Lobsters

Lobsters (1.25–1.50 lb.) used for technique-setup experiments were obtained from a commercial source located in Port Jefferson, New York. Lobsters used to investigate the effect of bacteria on ROS production were collected from traps deployed north of Oak Neck, Long Island Sound (Fig. 1). Animals were maintained in 200-L tanks (3 lobsters per tank), filled with aerated recirculating seawater containing artificial shelters made of PVC pipes. The water, which was maintained at  $32 \pm 1$  ppt and  $15 \pm 1^\circ C$ , was continuously pumped through a canister filter system containing active biofilter media made from sintered glass (Eheim Ehflüstrat, Aquatic Eco-Systems Inc., Apopka, Florida) and active carbon. Nitrate/nitrite and ammonia levels were monitored, and water changes were made when needed. Lobsters were fed twice a week, using clam meat for a minimum period of 7 days before bleeding and ROS measurement. To investigate ROS production in lobsters exposed to different levels of dissolved oxygen, additional lobsters

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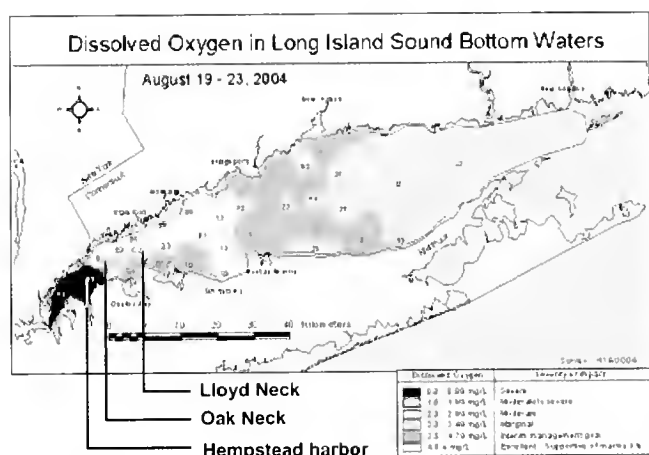


Figure 1. Map showing lobster sampling sites in Long Island Sound and dissolved oxygen in bottom waters during the sampling period (Map generated by CT DEP Long Island Sound Water Quality Monitoring Program, accessible online at: <http://www.dep.state.ct.us/wtr/lis/monitoring/summer2004.htm>)

were collected from different locations within Long Island Sound (Fig. 1): 12 lobsters were collected from traps located north of Hempstead Harbor, 15 from traps located north of Oak Neck, and 15 from traps located north of Lloyd Neck. These lobsters were maintained in seawater tanks onboard of the fishing vessel until transferred to the laboratory for immediate hemolymph sampling and processing.

#### Chemicals and Buffers

Crustacean anticoagulant (CAC, 0.45 M NaCl, 0.1 M glucose, 0.03 M trisodium citrate, 0.026 M citric acid, and 0.01 M EDTA, pH 4.6) and marine crustacean saline (MCS, 0.58 M NaCl, 0.013 M KCl, 0.013 M CaCl<sub>2</sub>, 0.026 M MgCl<sub>2</sub>, 0.00054 M N<sub>2</sub>HPO<sub>4</sub>, and 0.05 M Tris-HCL buffer, pH 7.6) solutions were prepared according to Smith and Söderhäll (1983). Dichlorofluorescein diacetate (DCFH-DA, Sigma) was dissolved in DMSO to obtain 100 mM stock solution (aliquoted and stored at -20°C). Work solutions of DCFH-DA were produced by diluting aliquots in filtered (0.22 µm) autoclaved seawater (FASW) at different concentrations as described later. Phorbol myristate acetate (PMA, Sigma) stock solution was made by dissolving 1 mg PMA in 1 mL dimethyl sulfoxide (DMSO). This solution was divided into 25-µL aliquots and stored at -20°C. Zymosan (Sigma) was directly suspended in FASW (20 mg 1 mL<sup>-1</sup>), then heated in a boiling water bath for 30 min. The resulting suspension was then washed twice by centrifugation and resuspension in MCS. The particle count was checked microscopically and aliquots were frozen at -20°C.

#### ROS Measurement

##### General Design

Hemolymph (0.5 mL) was withdrawn from the ventral sinus by inserting a needle at the base of the last left walking leg. Hemolymph was directly collected in a 3-mL syringe containing 2.5 mL CAC to prevent blood clotting. Diluted hemolymph was then centrifuged (300g, 4°C, 10 min). The supernatant was discarded, and the cells were resuspended in 1 mL MCS. An aliquot (100 µL) of resuspended hemocytes was added with formalin (2% final concentration) and used for hemocyte count. Remaining

hemocytes were added to a 96-well black microplate (100 µL per well, in 3–4 replicates depending on experiments) and were supplemented with DCFH-DA. An initial reading of the fluorescence (485 nm excitation and 535 nm emission) was then performed to measure the native (prior to activation) ROS production in hemocytes using a Wallace 1420 fluorometric plate reader. After the first reading, hemocytes were added with targeted activator (PMA or zymosan, see below), then additional fluorescence readings were recorded after 5 and 30 min of incubation in the dark.

#### Activation With PMA Versus Zymosan

Hemocytes resuspended in MCS were added to a 96-well microplate as follows: for each lobster ( $n = 3$  lobsters), nine replicate wells containing 100 µL per well were performed: 3 wells received 100 µL MCS containing 2 µL PMA stock solution (10 µg mL<sup>-1</sup> final concentration), another three received 100 µL Zymosan suspension made in MCS (1:50 hemocyte:zymosan ratio), and the last three received MCS as a control. Then 2 µL DCFH-DA stock solution were added to each well (final DCFH-DA concentration = 1 mM). Readings were taken using the Wallace fluorometric plate reader at 5, 20, 60, 90 and 120 min postactivation.

#### Determination of Optimal DCFH-DA Concentration

Hemocytes were processed in the same way as above. For each lobster ( $n = 3$ ), four replicate wells were performed, and each of these wells received a different concentration of DCFH-DA. The different concentrations tested were: 0.1, 0.5, 1, and 2 mM DCFH-DA. The Wallace plate reader was set up to take automatic readings at 5-min intervals for a total of 90 min after activation with zymosan (1:50 hemocyte:zymosan ratio).

#### Effect of Bacterial Challenge on ROS Production

The significance of pathogenic stress was investigated by studying the effect of *in vitro* challenge with bacterial compounds on ROS production in lobster hemocytes. Four bacterial strains were investigated: *Aerococcus viridans* var. *homari* (Robohm et al. 2005) and *Vibrio fluvialis* (Tall et al. 2003), both known to be pathogenic to lobsters, *Listonella anguillarum* (strain 775, Crosa et al. 1977), a pathogen to several marine organisms including fish and shellfish, and *Escherichia coli*, which is not known to cause disease in marine organisms (here used as control). Bacteria were cultured in sterile marine broth (Difco 2216) at room temperature on a shaker table for about 24 h (exponential phase of growth). After spectrophotometrically estimating bacterial counts, bacterial cells were collected by centrifugation (3,000g, 15 min, 4°C), resuspended in MCS and used immediately for challenge experiments. Bacterial supernatants were filtered (0.22 µm) and saved (-20°C) for use in subsequent experiments.

The first experiment investigated the effect of challenge of hemocytes with bacterial cells on ROS production using additional lobsters collected from Lloyd Neck. For each lobster (9 total), 12 wells received 100 µL of hemocytes resuspended in MCS, supplemented with DCFH-DA at a final concentration of 0.5 mM. After an initial reading to measure native ROS activity, duplicate wells received 20 µL of one of the following suspensions (at a ratio of 1:50 hemocytes:test particles, e.g., cfu or zymosan): *A. viridans*, *V. fluvialis*, *L. anguillarum*, *E. coli*, zymosan, and two wells received 20 µL MCS as a control. After a 30-min incubation in the dark, a second reading was taken, then 10 µL zymosan were added to each well and two more readings were taken after 5 and 30 min addi-

tional incubation. Aliquots of hemolymph samples were diluted in CAC, fixed with formalin (1.9% final concentration) and used for hemocyte counts with a hemocytometer. ROS production was normalized to hemocyte counts.

The second experiment investigated the effect of bacterial supernatants on ROS production by hemocytes. For each lobster (9 total), 12 replicated wells received 100  $\mu$ L of hemocytes resuspended in MCS. Duplicate wells were added with 100  $\mu$ L of bacterial (*A. viridans*, *V. fluvialis*, *L. anguillarum*, *E. coli*) supernatants. The volume of supernatant added per hemocyte was estimated to be equivalent to the volume of culture medium containing 50 cfu (supernatant-cfu equivalent). Two other wells received 100  $\mu$ L MCS as a control and two additional wells received 100  $\mu$ L sterile culture media as a second control. After 1-h incubation, DCFH-DA was added to each well at a final concentration of 0.5 mM and baseline fluorescence reading was taken. Then 10  $\mu$ L zymosan was added to each well and readings were taken 5 and 30 min after incubation in the dark. ROS production was normalized to hemocyte counts as described above.

#### Effect of Hypoxia on ROS Production

This experiment investigated ROS production in lobsters collected from areas on Long Island Sound known to be submitted to different levels of dissolved oxygen as determined by the Connecticut Department of Environmental Protection (CT DEP) Long Island Sound Water Quality Monitoring Program (Fig. 1). The biweekly summer survey generates hypoxia maps based on the monitoring of different stations across the sound using a CTD recorder. Lobsters were transferred to the laboratory and hemocytes were withdrawn as previously described. For each lobster, four wells received 100  $\mu$ L of hemocytes resuspended in MCS, supplemented with DCFH-DA at a final concentration of 0.5 mM. After an initial fluorometric reading to measure native activity, 10- $\mu$ L Zymosan suspension was added to each well to activate ROS production (about 1:50 hemocyte:zymosan ratio). Two additional readings were then made 5 and 30 min after incubation in the dark. ROS production was normalized to hemocyte counts as described for the bacterial challenge experiments.

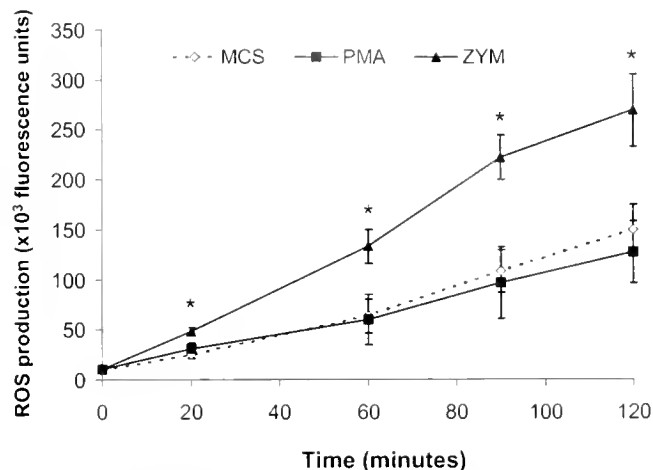
#### Statistics

Average fluorescence signals were calculated for all replicated wells before being processed for statistical analyses. All data were analyzed using the Statgraphics statistical software. Statistical tests were a one-way general linear model analysis of variance (ANOVA) or repeated measure ANOVA followed by a Fisher's LSD posthoc test, as appropriate. Because raw data were not always normally-distributed, a Log<sub>10</sub>-transformation was made on data before running statistical testing. Differences were considered significant at  $\alpha = 0.05$ .

## RESULTS

#### Activation of Hemocytes for ROS Production

The addition of 1 mM dichlorofluorescein diacetate (DCFH-DA) to hemocytes induced the production of baseline fluorescence that increased with incubation time, up to 120 min after incubation (maximal incubation period assayed in this study, Fig. 2). Because the fluorescence signal is an indicator of hydrogen peroxide production, these results suggest that ROS are produced in untreated hemocytes. Hydrogen peroxide production was significantly



**Figure 2.** Oxidative burst in hemocytes activated with phorbol myristate acetate (PMA) or zymosan (ZYM). Control hemocytes were incubated with saline (MCS). Fluorescence signals were significantly higher (\*) in hemocytes added with zymosan when compared with the 2 other treatments (Fisher's LSD posthoc test,  $P < 0.05$ ,  $n = 3$ ).

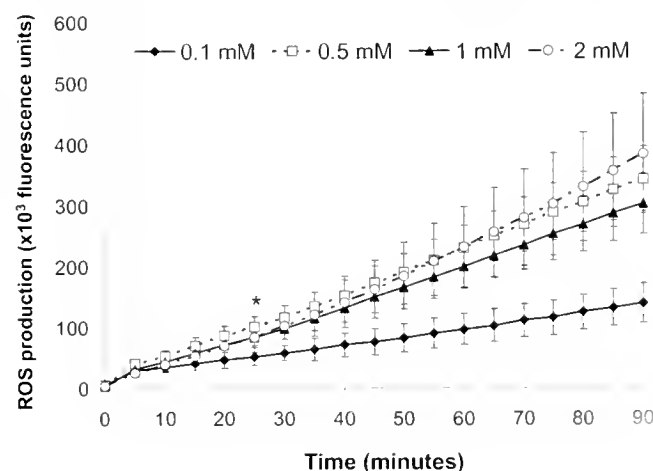
stimulated by the addition of zymosan A ( $P < 0.001$ , repeated measure ANOVA) within 20 min after activation, but was not modified by the addition of PMA, when compared with untreated hemocytes (added with saline). These trends were not modified over the 2-h duration of the experiment.

#### Determination of Optimal DCFH-DA Concentration

Fluorescence signals intensified with the increase of DCFH-DA concentration from 0.1–0.5 mM (Fig. 3). Further increase in concentration to 1 or 2 mM did not enhance the intensity or modify the kinetics of H<sub>2</sub>O<sub>2</sub> production. Based on these results, a concentration of 0.5 mM was used in all subsequent experiments.

#### Effect of Bacterial Cells on the Oxidative Burst

Thirty minutes after the addition of bacterial cells to hemocytes, ROS production significantly increased in wells incu-



**Figure 3.** Oxidative burst in hemocytes added with different concentrations of dichlorofluorescein diacetate (DCFH-DA) and activated with zymosan. \*: significant differences between hemocytes added with 0.1 mM and all other treatments appeared 25 min after incubation and continued until the end of the experiment (Fisher's LSD posthoc test,  $P < 0.05$ ,  $n = 3$ ).

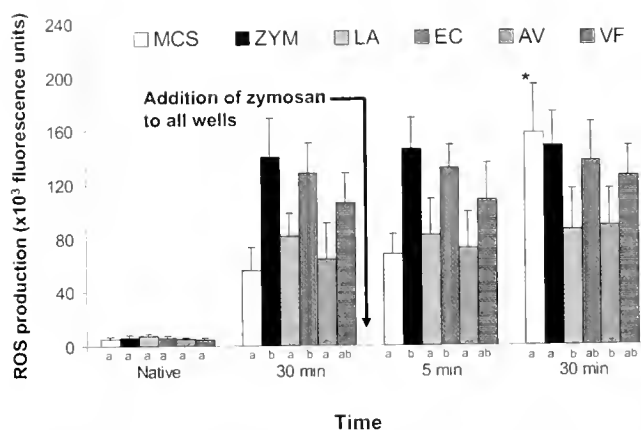


Figure 4. Effect of bacterial cells on reactive oxygen species production in lobster hemocytes. Zymosan was added to all wells after the second fluorescence reading. MCS: saline, ZYM, zymosan, LA: *Listonella anguillarum*, EC: *Escherichia coli*, AV: *Aerococcus viridans*, VF: *Vibrio fluvialis*. For each time interval, letters (a and b) represent significant differences among different treatments (Fisher's LSD post hoc test,  $P < 0.05$ ,  $n = 9$ ). \*: significant increase when compared with the prior sampling point (Student  $t$ -test,  $P < 0.05$ ).

bated with *E. coli*, when compared with control hemocytes added with saline (Fig. 4). The other bacterial species caused only a slight increase in fluorescence signals that did not significantly differ from control hemocytes. Among this group, *V. fluvialis* caused the highest signals, followed by *L. anguillarum* and finally by *A. viridans*. The subsequent activation of hemocytes with zymosan did not significantly change the trends observed in hemocytes preincubated with bacteria. The major changes were observed in hemocytes initially used as controls (added with saline), in which the addition of zymosan produced the strongest signals (Fig. 4). A second addition of zymosan to hemocytes did not noticeably increase ROS production.

#### Effect of Bacterial Extracellular Products on the Oxidative Burst

Pre-incubation of hemocytes with bacterial extracellular products (ECP) did not affect native hydrogen peroxide production in

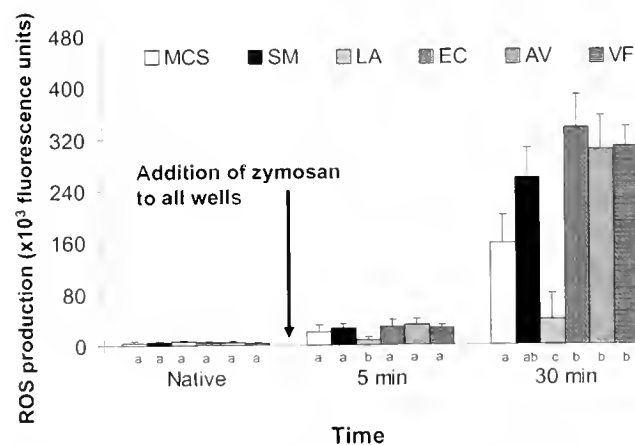


Figure 5. Effect of bacterial extracellular products (ECP) on reactive oxygen species production in lobster hemocytes. See legend of Figure 4 for abbreviations. For each time interval, letters (a, b and c) represent significant differences among different treatments (Fisher's LSD posthoc test,  $P < 0.05$ ,  $n = 9$ ).

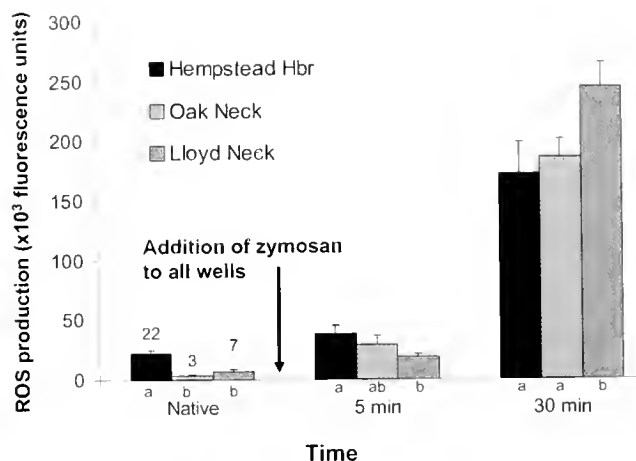


Figure 6. Reactive oxygen species production in lobsters collected from three different locations in Long Island Sound (see Figure 1 for more details). Each data point represents 12 (Hempstead Harbor) or 15 (Oak Neck Harbor and Lloyd Neck) lobsters. Letters (a and b) represent significant differences among samples (Fisher's LSD posthoc test,  $P < 0.05$ ).

inactivated hemocytes. However, hemocytes preincubated with ECP from *L. anguillarum* were unable to express oxidative bursts in response to zymosan activation (Fig. 5). Preincubation of hemocytes with ECP produced by the other bacterial species slightly increased ROS production when compared with saline control. However, ROS signals in these samples were not significantly different from those of control hemocytes incubated in sterile bacterial culture media.

#### Effect of Hypoxia on ROS Production

Native hydrogen peroxide production in inactivated hemocytes was significantly higher in lobsters collected from the most western field site (Hempstead Harbor), when compared with lobsters from Oak Neck and Lloyd Neck (Fig. 6). This trend is inverted after activation. For instance, thirty minutes after the addition of zymosan, oxidative burst was significantly higher in lobsters collected from the most eastern site when compared with the two other locations. Hemocyte counts were not significantly different among the different field sites and ranged from  $1.9 \times 10^6$  to  $4.8 \times 10^6$  hemocytes  $\text{mL}^{-1}$ .

#### DISCUSSION

Oxidative burst by blood cells is a major antimicrobial mechanism in vertebrates and invertebrates. This oxidative process starts when stimulation of macrophages leads to increased consumption of oxygen, the reduction of which, catalyzed by a membrane-bound NADPH oxidase, initiates the cascade and production of several reactive oxygen species. This study demonstrates that the optimized fluorometric technique represents a valuable method for the measurement of oxidative burst by lobster hemocytes, and provides a viable means of evaluating the effects of pathological and environmental stresses on lobster immunity. Our results show that dichlorofluorescein-diacetate (DCFH-DA) is cleaved into fluorescent dichlorofluorescein in hemocytes incubated in saline at a significantly lower rate than in hemocytes added with zymosan. Higher cleavage rate clearly indicates a higher production of hydrogen peroxide through activation of the ROS cascade (Rosen-



kranz et al. 1992). The fluorescent signal increased with incubation time, including that in control hemocytes, as a result of accumulation of dichlorofluorescein in cells. Fluorescent signals observed in control hemocytes correspond to spontaneous  $H_2O_2$  production, which may reveal a normal physiological cell activity. Alternatively, such production can be related to an activation of hemocytes during the isolation procedure, or when the hemocytes adhere to the plastic as observed, for instance, for bivalve (Pipe 1992) or hypothesized for crustacean (Bachère et al. 1995) hemocytes. Incubation of hemocytes with phorbol myristate acetate (PMA,  $10 \mu\text{g mL}^{-1}$ ) did not induce any increase in hydrogen peroxide production. This is in agreement with several studies that found that PMA is not as efficient in inducing ROS production as zymosan in crustaceans (Song & Hsieh 1994, Bachère et al. 1995, Muñoz et al. 2000) and in mollusks (Ordas et al. 2000, Austin & Paynter 1995, Torreilles et al. 1997), but in disagreement with a prior report that showed higher ROS production in lobster (*H. americanus*) hemocytes activated with PMA, when compared with cells added with zymosan (Anderson & Beaven 2005). This apparent contradiction could be explained by the fact that Anderson & Beaven measured the production of a subsequent reactive intermediate in the cascade, namely  $HOCl$ , using a chemiluminescence detection method. The fact that the hemocyte:zymosan ratio used here (1:50) is significantly higher than ratios used by those authors (1:2–1:25) may provide another explanation of the differences observed between the two studies, because our preliminary results showed an increase in hydrogen peroxide production with the increase of hemocyte:zymosan ratio up to 1:50 (data not shown).

The possible role of lobster hemocyte ROS in the elimination of pathogenic microorganisms was investigated by studying the effect of selected bacteria on ROS production *in vitro*. Our results demonstrated that *E. coli* and *V. fluvialis* cells caused an increase in ROS production, but differences compared with the control were only significant in the case of *E. coli*. Although a prior report also demonstrated that *E. coli* cells induced the highest ROS response in shrimp hemocytes when compared with shrimp bacterial pathogens (Muñoz et al. 2000), the biological significance of these results is not clear, because *E. coli* is not significantly present in natural lobster habitat. It seems, however, that some bacterial pathogens have developed strategies to avoid the trigger of ROS production by hemocytes. For instance, Muñoz et al. (2000) demonstrated that a pathogenic strain of *Vibrio harveyi* did not induce ROS production when added to shrimp hemocytes, whereas the addition of a strain of *V. alginolyticus* used as probiotic in shrimp aquaculture did enhance ROS production. Similarly, our study revealed that *Aerococcus viridans*, a pathogen that is able to survive phagocytosis by lobster hemocytes (Stewart 1975), does not elicit ROS production. Similarly, the universal pathogen *L. anguillarum* did not induce the production of ROS. More importantly, ROS production in these two samples was not increased after the addition of zymosan, suggesting an active neutralization of the oxidative metabolism by *A. viridans* and *L. anguillarum*. Although bacterial cells from both strains have similar effects, the mechanisms underlying their ROS inhibition might be different. It has already been demonstrated that the survival in hosts' cells of pathogenic bacteria and fungi is often linked to the ability of these microorganisms to quench ROS through the production of catalases/peroxidases, which are thus considered virulence factors (Day et al. 2000, Lefebvre & Valvano 2001, Pongpom et al. 2005). It is thus possible that *A. viridans* quenches ROS production in

lobster hemocytes as a mean to colonize and survive within host's cells. The antioxidant enzyme catalase is not present in this bacterial species, however, suggesting that such quenching might be performed through other processes. The effect of *L. anguillarum* on ROS production is more pronounced when bacterial extracellular products (ECP) are used. *L. anguillarum*, which possesses catalase activity, may suppress fluorescence signals by altering  $H_2O_2$  production as suggested by Bramble and Anderson (1997). Alternatively, the same strain of this universal pathogen has demonstrated wide cytotoxic effects on hemocytes from different bivalve species under similar experimental conditions (Allam & Ford 2006). The viability of hemocytes was not specifically monitored in the present work, but anecdotal observations suggested increased mortality only in hemocytes incubated with *L. anguillarum* (data not shown). The effect of *L. anguillarum* observed here might simply be a result of hemocyte killing by bacterial cells and ECP, because dead hemocytes could not participate in ROS production.

The comparison of ROS production in lobsters collected from field sites submitted to different environmental conditions suggests that this technique represents a good indicator of lobster physiological status and immunocompetency. The major environmental condition that varies among experimental sites was the level of dissolved oxygen, which ranged from levels below  $1 \text{ mg L}^{-1}$  in Hempstead harbor to about  $3 \text{ mg L}^{-1}$  in Lloyd Neck. Native hydrogen peroxide production was 3 times higher in hemocytes from lobsters collected in the former, when compared with those harvested from the latter. High native (base) ROS production may reveal stressful or injurious situations caused by *in situ* factors. These findings suggest that lobsters from Hempstead harbor had been submitted to oxidative stress, which may have been caused by low oxygen availability in the environment (Storey 1996, Pan et al. 2003). Animals typically respond to hypoxia by reducing metabolic rate (Perez-Rostro et al. 2004), but side effects of hypoxia include the formation of excess free oxygen radicals (Yu 1994, Pan et al. 2003), causing severe alterations in cellular activities. Although these microbiocidal agents are typically generated in the phagocytic vacuoles, an important quantity crosses into the extravacuolar environment and may cause damage to cells (Warner 1994, Thompson et al. 1995). ROS also damage cytoskeletal proteins, leading to a probable loss in defense function, because hemocyte adhesion, motility, endocytic ability and the capacity to phagocytose foreign particles all depend on the cytoskeleton (Bellomo & Mirabelli 1992). The prevention of such damages is normally made through different defense strategies, which involve the use of small antioxidant molecules (ascorbate, glutathione, carotenoid pigments) that can directly neutralize oxidative radicals, or rely on a variety of enzymes that metabolize ROS (superoxide dismutase, catalase, glutathione peroxidase) (Warner 1994). In any case, subsequent stimulation with a specific trigger of the respiratory burst could be impaired, ineffective or unreliable. For instance, hemocytes from hypoxic areas (Hempstead and Oak Neck) did not respond to zymosan activation as efficiently as those collected from Lloyd Neck. Similarly, Le Moullac et al. (1998) demonstrated a decrease in ROS production, measured by the nitroblue tetrazolium method, in shrimps submitted to hypoxia. In our study, the lowering of ROS production may hamper the lobster's ability to mount an effective defense against invading microorganisms in areas affected with hypoxia, thus amplifying stress to such animals and leading to mass mortality events such as those that have been observed in western Long Island Sound.

In conclusion, this paper describes a simple technique to measure oxidative burst in lobster hemocytes. The technique was used to demonstrate a deleterious effect of some bacterial pathogens on ROS production in lobster. Additionally, the technique seems promising for field monitoring of lobster immunocompetency. Our results suggest that pathogen and environmental stresses may combine and act together to create potentially disastrous conditions for lobsters.

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## AN IMPROVED AND RAPID BIOCHEMICAL IDENTIFICATION OF INDIGENOUS AEROBIC CULTURABLE BACTERIA ASSOCIATED WITH GALICIAN OYSTER PRODUCTION

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**ABSTRACT** An improved and rapid biochemical identification of the culturable aerobic bacteria species associated with Galician oyster production was performed by the combination of numerical taxonomy and sequencing of 16S rRNA gene. The 16S rRNA sequences of 23 representative bacterial isolates from different growing stages of *Ostrea edulis*, surrounding water, and phytoplankton were compared with related sequences from the EMBL database. These results were used to identify the phenetic clusters obtained by numerical taxonomy using the *S*<sub>J</sub>/UPGMA with a similarity level of 74%. The combination of the two techniques was a useful tool for identifying 40 out of 75 representative aerobic Gram negative isolates comprising the bacterial community studied and for improving the phenotypical description of each identified species. It was mostly facultative psychrophilic  $\gamma$ -*Proteobacteria* showing a great diversity. No specificity of bacteria, according to the geographical area studied was found. Almost all of the identified species were associated, for the first time, with different growing stages of oyster. Some could have a probiotic effect (*Roseobacter gallaeciensis*, *Shewanella schlegeliana*) or could be a potential risk for oyster cultures (*Pseudoalteromonas piscicida*, *Pseudomonas anguilliseptica*) or for humans by consumption (*Acinetobacter johnsonii*, *Pseudoalteromonas tetraodonis*).

**KEY WORDS:** oyster, culturable aerobic bacteria, 16S rRNA gene, rapid identification

### INTRODUCTION

The main culture of bivalve mollusc volume in Europe is concentrated in Galicia. Maintaining cultures with a high density increases the risks and consequences of infectious outbreaks. The study of natural culturable microbiota is essential for improving the industrial culture production, because it is the first step for designing a rapid presumptive guide for separating environmental and potentially pathogenic bacteria species. In a previous paper, we reported the phenotypical analyses of culturable aerobic bacteria associated with the Galician oyster (*Ostrea edulis*) cultures (Guisande et al. 2004). The samples were obtained monthly from oyster culture systems located on the Galician coast at: Bueu, Couso, Grove, Malpica, Ribadeo, and Vilagarcía de Arousa over 12 consecutive months, as was previously reported. A total of 397 isolates from different stages (seed, larval, and reproductive) of oyster, surrounding water and phytoplankton, selected as being representative of bacterial community, were characterized by numerical taxonomy. Nineteen per cent of isolates (75) were aerobic, gram negative, and using the Jaccard's coefficient at 69% (*S*<sub>J</sub>) and the unweighted pair group average method (UPGMA) a great diversity, and a high number of unidentified isolates were obtained.

The aim of our study was to select differential biochemical tests for a rapid identification of isolates after the identification by using the sequencing of 16S rRNA gene technique and numerical taxonomy analysis. A phenotypic description of each identified species will be provided. This study will differentiate between the strains associated with growing oyster stages, with a probiotic role or potentially pathogenic species for oysters or humans.

### MATERIALS AND METHODS

#### Bacterial Strains

A total of 75 aerobic strains were obtained as representatives on a wide study of bacterial community associated with Galician oyster production from different stages (seed, larval, and reproduc-

tive) of oyster (*Ostrea edulis*), surrounding water, and phytoplankton (Guisande et al. 2004). Sampling, processing, and isolation of strains were previously reported by us. Pure cultures of strains were obtained on Marine Agar (Cultimed, Barcelona, Spain) and were stored at -80°C in Nutritive Broth (Cultimed, Barcelona, Spain) with 2% (w/v) NaCl (Pancreac, Barcelona, Spain) and 15% (v/v) of glycerol (Panreac).

#### Sequencing of 16S rRNA Gene

Sequencing of 23 strains (Table 1) representing all phenotypes and some of unclustered strains of dendrogram previously reported by us (Guisande et al. 2004) was performed according to the method described by Montes et al. (2003) using a 310 Genetic Analyzer (Applied Biosystems, Darmstadt, Germany) automated sequencer. The sequence of the 16S rRNA gene of strains was determined by using four primers (37F, 344F, 344R, and 1096R) and compared with sequences in public databases of GenBank, EMBL and DDBJ with BLAST, version 2.2.6. (Altschul et al. 1997). Multiple alignment of sequences was created by ClustalX, version 1.81 (Higgins & Sharp 1988), which included 1003 positions after removal of ambiguous positions (Hall 2001), using the BioEdit Sequence Alignment Editor, version 5.0.9 (Hall 1999). A phylogenetic tree was constructed by using Molecular Evolutionary Genetics Analysis (MEGA), version 2.1 (Kumar et al. 2001). This was performed using the neighbor-joining method (Saitou & Nei 1987) and Tamura-Nei distance model (Tamura & Nei 1993), with the calculation of cluster stability by bootstrap analysis with 1,000 replicates (Nei & Kumar 2000).

#### Sequences of Organisms Used for Phylogenetic Trees

All available species of each genus closest to studied strains were selected. In most cases, species type strains (if available) with nearly full-length 16S rRNA sequences were used. This includes members of two phylogenetic groups: Proteobacteria- $\gamma$  subdivision: *Acinetobacter* (16 species), *Alteromonas* (3 species; it was abbreviated as *Alt.*), *Halomonas* (27 species), *Marinobacter* (7 species), *Mesorhizobium* (5 species), *Pseudomonas* (29 species), *Pseudoalteromonas* (24 species; it was abbreviated as *Pa.*) and

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TABLE 1.

Identification and origin of aerobic bacteria species associated with Galician oyster production on the basis of sequencing of the 16S rRNA gene and numerical taxonomy.

Species	Origin of isolates				
	Oyster ( <i>Ostrea edulis</i> )			Seawater	Phytoplankton
	Seed	Larval	Reproductive		
<i>Acinetobacter</i>					
<i>A. johnsonii</i>	—	+ (31.98c, 32.98c)	+ ( <b>42b</b> )	+ (16.98f)	—
<i>Alteromonas</i>					
<i>Alt. macleodii</i>	—	+ (17.98f, 622e, <b>642e</b> )	—	+ ( <b>29.98f</b> , 30.98f)	+ (50.98d)
<i>Halomonas</i>					
<i>H. venusta</i>	—	—	—	+ (57.98d, 59.98d)	+ ( <b>51.98d</b> )
<i>Marinobacter</i>					
<i>M. flavimaris</i>	+ (14.98c)	+ (86.98b, <b>73.98f</b> )	—	—	+ ( <b>52.98d</b> )
<i>Pseudoalteromonas</i>					
<i>Pa. piscicida</i>	—	+ (61.98d)	+ (304.98b)	+ ( <b>55.9 8d</b> )	—
<i>Pa. tetraodonis</i>	—	+ ( <b>82.98b</b> )	—	—	—
<i>Pa. undina</i>	+ ( <b>251c</b> )	+ ( <b>131b</b> )	—	—	—
<i>Pseudomonas</i>					
<i>P. anguilliseptica</i>	—	+ ( <b>173c</b> )	—	—	—
<i>P. mendocina</i>	—	+ ( <b>170c</b> , <b>174c</b> )	—	—	—
<i>Roseobacter</i>					
<i>R. gallaeciensis</i>	+ ( <b>155c</b> )	—	—	—	—
<i>Shewanella</i>					
<i>S. japonica</i>	—	—	+ (80b, <b>86b</b> , 87b)	—	—
<i>S. livingstonensis</i>	—	+ ( <b>171c</b> )	—	—	—
<i>S. pacifica</i>	—	+ ( <b>111b</b> )	—	—	—
<i>S. schelegeliana</i>	—	—	+ ( <b>81b</b> )	—	—
<i>S. waksmanii</i>	—	+ (297.98b)	+ ( <b>117b</b> , 120b, 123b, 549a, 560c, <b>561c</b> )	—	—

In brackets: strain plus geographical area (a, Bueu; b, Couso; c, Grove; d, Malpica; e, Ribadeo; f, Vilagarcia). In bold print: selected strains for sequencing of 16S rRNA analysis.

*Shewanella* (33 species), and Proteobacteria- $\alpha$  subdivision with 3 species of *Roseobacter*.

#### Nucleotide Sequence Accession Numbers

The partial 16S rRNA sequences of environmental isolates reported in this paper (42, 642, 29.98, 51.98, 73.98, 52.98, 55.98, 82.98, 251, 131, 173, 170, 174, 155, 86, 171, 111, 81, 117, 561, 8.98, 34.98, 38.98) have been deposited in the DDBJ (Mishima, Japan), EMBL (Heidelberg, Germany) and GenBank (Mountain View, USA) nucleotide sequence data bases under accession numbers AY870662 to AY870684, respectively.

#### Phenotypic Characterization

Bacterial strains and the reference strains were previously characterized by 92 physiological, morphological, and biochemical tests (Guisande et al. 2004). Cultures grown during 24 h at 22°C on Tryptic Soy Agar (TSA, Cultimed) and supplemented up to 2% (w/v) NaCl (Panreac) (TSA 2%) were used as inocula. Data were processed with the NTSYS-pc, version 1.8 (Rohlf 1994). A similarity matrix was calculated using the  $S_j$  at 74%. Phenotypes were clustered using the UPGMA method and were defined with the earlier mentioned percentage. The correlation between the dendrogram and the similarity matrix (cophenetic correlation) was determined using the cophenetic correlation coefficient ( $r$ ). The reproducibility of the tests was evaluated by analyzing 10% of strains in duplicate, as suggested by Sneath and Johnson (1972).

The reference strains included in the numerical taxonomy study

were: *Achromobacter denitrificans* CECT (Colección Española de Cultivos Tipo, Valencia, Spain) 449<sup>T</sup>, *Agrobacterium ferrugineum* CECT 4356<sup>T</sup>, *Halomonas aquamarina* CECT 5000<sup>T</sup>, *Marinomonas communis* CECT 5003<sup>T</sup>, *Marinomonas vaga* CECT 5004<sup>T</sup>, *Marinobacter hydrocarbonoclasticus* CECT 5005<sup>T</sup>, *Porphyrobacter sanguineus* CECT 4271<sup>T</sup>, *Pseudoalteromonas* (abbreviated as *Pa*) *citrea* CECT 575<sup>T</sup>, *Pa. espejiana* CECT 5002<sup>T</sup>, *Pa. haloplanktis* CECT 4188<sup>T</sup>, *Pa. undina* CECT 5006<sup>T</sup>, *Pseudomonas fluorescens* CECT 378<sup>T</sup>, *Pseudomonas putida* CECT 324<sup>T</sup>, *Shewanella hanedai* CECT 5194<sup>T</sup>, and *Stappia aggregata* CECT 4269<sup>T</sup>.

## RESULTS

#### Sequencing of 16S rRNA Gene

The phylogenetic affiliation using the 16S rRNA sequence analysis was performed on 23 strains isolated from different growing stage of *Ostrea edulis*, surrounding water, and phytoplankton. They were representative within each phenon and some of them from unclustered strains of the previously reported dendrogram (Guisande et al. 2004). For most strains, sequences of the 16S rRNA gene, stretching from nucleotide positions 37–1040 (*Escherichia coli* equivalent), were obtained. These sequences were compared with each other and to related sequences, from the EMBL database, described in Material and Methods. The closest-neighboring species, which shared a similarity value in the 16S rRNA sequences of  $\geq 98\%$ , were used to identify the isolates. This

led to the identification of 20 strains from 23 selected strains, most of the identified strains belonging to the  $\gamma$ -*Proteobacteria*. Only 3 were not identified, indicating species that have not been sequenced previously. So, the strains 8.98, 34.98, and 38.98 showed pair-wise sequence similarities of less than 95% to their nearest validly named neighbors (Fig. 1) and would probably correspond to new species.

The sequenced strains, geographical areas, and origin of identified species are shown in Table 1.

#### Phenotypic Characterization

The simplified dendrogram of aerobic strains showing the phenotypes defined with a value of  $S_j$  of 74% are presented in Figure 1. The average probability ( $P$ ) of an erroneous test result (0.02) and the cophenetic correlation coefficient ( $r$ ) (0.95) were acceptable values. The 60.44% (55/91) of strains were unclustered, and all the species identified by 16S rRNA sequence analysis were grouped into different phenetic clusters or were unclustered. Using the sequence of 16S rRNA gene 40 strains from 75 analyzed were identified. *Pa. undina* and *P. mendocina* included strains that were ungrouped. The strains identified as *Alt. macleodii*, *M. flavimaris*, and *S. waksmanii* were also grouped into separate phenotypes (Fig. 1), confirming the presence of various biotypes in each of those species. The isolates identified as *A. johnsonii*, *H. venusta*, *Pa. piscicida*, and *S. japonica* were each included in one phenotype, showing a high phenotypic homogeneity. All these aerobic strains were heterotrophic facultative psychrophilic, showing the same response for 49 tests (from the 87 analyzed), as previously described by Guisande et al. (2004). Characteristics not previously reported and differential tests of the identified species were shown in Table 2. From the 39 new reported tests, 35 were discriminatory to identify species. A selection of 4–15 tests for rapid identification of each species was made after developing a dichotomic differential table of species (Table 2).

#### DISCUSSION

The 16S rRNA sequence analysis has been used as a successful tool for identifying new strains (Stackebrandt & Goebel 1994, Wiik et al. 1995, Patel et al. 1998, Farto et al. 2003, Montes et al. 2003) and for identifying indigenous bacteria population isolated from the natural environment (González & Moran 1997, Kirchman 2002, Schauer et al. 2003). To clarify the taxonomic status of 75 representative aerobic isolates and select differential biochemical tests for a rapid identification of isolates from oyster culture, the phylogenetic affiliation using the 16S rRNA sequence analysis was performed on 23 strains. These strains were representative within each phenotype, and some of them were from unclustered strains. The results showed that some of the isolates identified by sequencing as different species were grouped in the same phenetic cluster, making it difficult to obtain a reliable identification. To establish the phenetic clusters including only one species identified by 16S rRNA sequence analysis, we grouped the aerobic strains of our previous work (Guisande et al. 2004) by numerical taxonomy with a higher  $S_j$  (74%) (Fig. 1). These results confirmed a high diversity of aerobic bacteria, as in Mediterranean oyster (*Ostrea edulis*) (Pujalte et al. 1999). The 16S rRNA sequence analysis led to the identification of 20 strains from 23 selected strains and 40 strains from 75 analyzed in this study (Table 2). Thus, both methods, numerical taxonomy and 16S rRNA sequences, were necessary for identification. The first one provided groups of strains

with similar phenotypic characteristics (phenon) and the selection of representative strains within each phenotype. The second method led to the molecular identification of each phenetic cluster by the selection of representative strains within each phenotype. The combination of both techniques is a useful tool for identifying culturable isolates from unknown habitats with a great diversity. Most of the 16S rRNA sequencing data revealed the close phylogenetic relationship above the level proposed as the intraspecific variability ( $\geq 98\%$ , Stackebrandt & Embley 2000), and the identification was performed according to the highest phylogenetic similarity value among the sequence of isolates and the reference strains. Moreover, the high number of bases obtained and the bootstrap analysis with 1,000 replicates were used for the identification because of the reliable results it gives and the positive outcome that it has had in phylogenetic studies (Ivanova et al. 2002, Hayashi et al. 2003, Satomi et al. 2003, Thompson et al. 2003, Yoon et al. 2003).

Among the more abundant microbiota associated with Canadian oysters (*Crassostrea virginica*) and Pacific oysters (*Crassostrea gigas*), the genera *Pseudomonas*, *Shewanella* and *Acinetobacter* (Kueh & Chan 1985, Hariharan et al. 1995) were identified by phenotypical tests. We also found these genera in this work (Table 1), however, they were different from those associated with the Mediterranean oyster (*Ostrea edulis*), which were identified by hybridization with phylogenetic probes complementary to conserved regions of 16S rRNA (Pujalte et al. 1999). Unlike other studies, this study is the first research that includes the identification of culturable aerobic microbiota obtained from different stages of *Ostrea edulis* (seed, larval, and reproductive stage). In some cases, the same species was isolated from different stages of oyster and geographical area such as *A. johnsonii*, *M. flavimaris*, *Pa. piscicida*, *Pa. undina*, and *S. waksmanii*, confirming the non-specificity of these species (Table 1). Among the species previously described as isolated from seawater, we have identified at different stages of oyster *Alt. macleodii*, *M. flavimaris*, *Pa. undina*, *S. japonica*, and *S. pacifica* (Gauthier et al. 1995, Ivanova et al. 2001, 2004, Yi et al. 2004, Yoon et al. 2004). Another species typically isolated from sediment, which we identified from oyster larva was *S. livingstonensis* (Bozal et al. 2002). Some other strains identified could be a potential risk for oyster cultures, because those species were associated with fish deaths, such as *Pa. piscicida* (Bein 1954), *P. anguilliseptica* (Doménech et al. 1997) or for human disease such as *A. johnsonii*, usually considered an opportunistic pathogen (Towner 1997, Levi & Rubinstein 1996), or *Pa. tetradonis* that produce a tetrodotoxin (Simidu et al. 1990). We also identified species that could have a probiotic effect on oyster, such as *Roseobacter gallaeciensis* (Ruiz Ponte et al. 1999) and *S. schlegeliana* (Satomi et al. 2003), which were associated with a specific stage of oyster. Its potential use as probiotic should be tested. Finally, we also identified strains such as *P. mendocina* suggesting an earth contamination by soil (Satomi et al. 2003) or *S. waksmanii* showing the colonizing ability of different marine organisms, because other authors reported the association of this species with spicula (Ivanova et al. 2003). Thus, this is the first identification of these species associated with different growing stages of oyster.

Among the species isolated from seawater, we found *A. johnsonii*, *Alt. macleodii*, *H. venusta*, and *Pa. piscicida*. Other species of these genera were previously identified from seawater associated with the culture of *Ostrea edulis* in the Mediterranean by molecular methods (Pujalte et al. 1999). *Alt. macleodii*, *H. venusta*, and

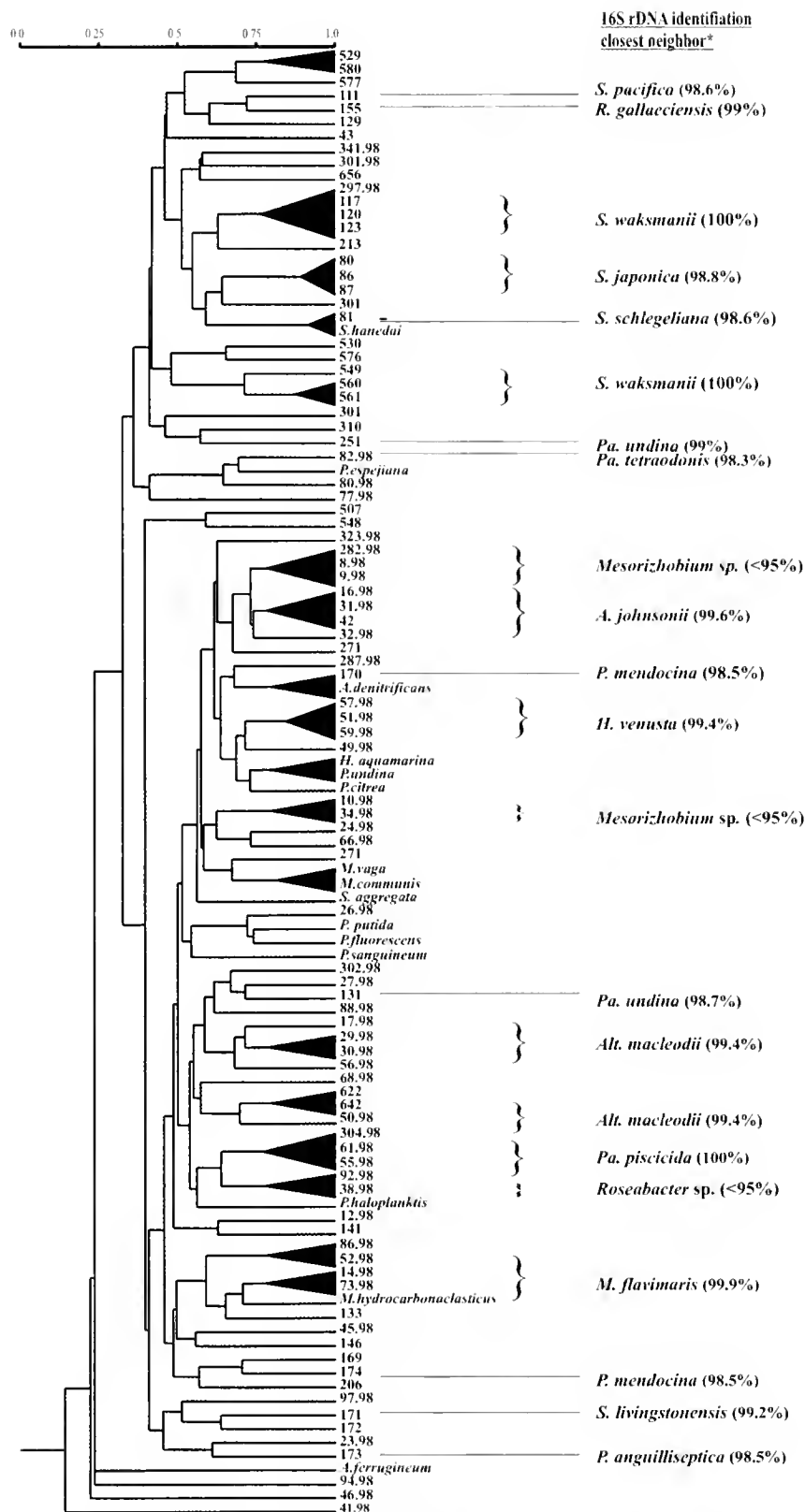


Figure 1. Simplified dendrogram of aerobic strains showing the phena defined with a value of  $S_J > 74\%$  and 16S rRNA identification (closest neighbor)<sup>1</sup>

\*The most closely related validly described species, in terms of 16S rRNA sequence. In brackets is shown the sequence similarity (%) of the isolate analyzed with the closest neighbor. <sup>1</sup>This dendrogram includes all aerobic strains reported by Guisande et al. 2004.



TABLE 2.  
Characteristics not previously reported and differential tests of aerobic species identified

Test	Aerobic species identified*														
	1 <sup>†</sup>	2 <sup>‡</sup>	3 <sup>‡</sup>	4 <sup>‡</sup>	5 <sup>†</sup>	6 <sup>  </sup>	7 <sup>  </sup>	8 <sup>  </sup>	9 <sup>  </sup>	10 <sup>  </sup>	11 <sup>†</sup>	12 <sup>  </sup>	13 <sup>  </sup>	14 <sup>  </sup>	15 <sup>‡</sup>
**	4	6	3	4	3	1	2	1	2	1	3	1	1	1	7
<sup>1</sup> ADH <sup>  </sup>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Glucose oxidation	(—)	—	—	—	—	—	—	—	—	—	— <sup>δ</sup>	— <sup>δ</sup>	— <sup>δ</sup>	— <sup>δ</sup>	— <sup>δ</sup>
<sup>1</sup> KIA/H <sub>2</sub> S	(—)	—	—	—	—	—	—	—	—	—	+ <sup>δ</sup>	— <sup>δ</sup>	—	— <sup>δ</sup>	+ <sup>δ</sup>
Nitrate reduction	v	(—)	+ <sup>δ</sup>	+ <sup>δ</sup>	— <sup>δ</sup>	—	— <sup>δ</sup>	+ <sup>δ</sup>	+ <sup>δ</sup>	— <sup>δ</sup>	+ <sup>δ</sup>	+ <sup>δ</sup>	+ <sup>δ</sup>	+ <sup>δ</sup>	+ <sup>δ</sup>
Growth at:															
4 °C	(+)	— <sup>δ</sup>	+ <sup>δ</sup>	— <sup>δ</sup>	— <sup>δ</sup>	+ <sup>δ</sup>	+ <sup>δ</sup>	+ <sup>δ</sup>	— <sup>δ</sup>	+ <sup>δ</sup>	+ <sup>δ</sup>	+ <sup>δ</sup>	+ <sup>δ</sup>	+ <sup>δ</sup>	(—) <sup>δ</sup>
10 °C <sup>  </sup>	+	(—)	+	v	+	+	+	+	+	+	+	+	+	+	+
37 °C	(+)	—	+ <sup>δ</sup>	+ <sup>δ</sup>	—	—	—	— <sup>δ</sup>	+ <sup>δ</sup>	—	—	—	—	—	— <sup>δ</sup>
44 °C <sup>  </sup>	(—)	—	—	v	—	—	—	—	—	—	—	—	—	—	—
pH 10	+ <sup>δ</sup>	— <sup>δ</sup>	+ <sup>δ</sup>	+ <sup>δ</sup>	+ <sup>δ</sup>	— <sup>δ</sup>	+ <sup>δ</sup>	+ <sup>δ</sup>	+ <sup>δ</sup>	+ <sup>δ</sup>	+ <sup>δ</sup>	+ <sup>δ</sup>	+ <sup>δ</sup>	+ <sup>δ</sup>	+ <sup>δ</sup>
Growth in:															
0.5% NaCl	+	(—)	+	v	v	—	— <sup>δ</sup>	+	+	+ <sup>δ</sup>	—	+ <sup>δ</sup>	—	— <sup>δ</sup>	— <sup>δ</sup>
5% NaCl	+	+	+	+	+	+	v	—	+	+	v	+	+	—	(—)
7% NaCl	+	+	+	+ <sup>δ</sup>	v	+	v	—	— <sup>δ</sup>	—	—	—	—	—	—
10% NaCl	(—)	v	+ <sup>δ</sup>	(+)	—	—	v	—	—	—	—	—	—	—	—
Crystal violet	(—)	(—)	+ <sup>δ</sup>	(—)	v	—	v	+ <sup>δ</sup>	+	—	— <sup>δ</sup>	— <sup>δ</sup>	— <sup>δ</sup>	— <sup>δ</sup>	(+) <sup>δ</sup>
<sup>1</sup> TCBS Agar	—	—	—	—	—	—	v	—	—	+ <sup>δ</sup>	+	—	—	—	(+)
<sup>1</sup> TCBS (yellow)	—	—	—	—	—	—	— <sup>δ</sup>	—	—	+ <sup>δ</sup>	+ <sup>δ</sup>	—	—	—	— <sup>δ</sup>
Acid from:															
D-galactose	(—)	—	—	—	nd	nd	nd	+ <sup>δ</sup>	—	+	—	—	—	—	— <sup>δ</sup>
D-mannose	v	—	—	—	nd	nd	—	—	—	—	—	—	+	—	—
Degradation of:															
Starch	—	+	—	(—)	+	+	v	—	v	+	+ <sup>δ</sup>	—	+	—	— <sup>δ</sup>
Esculin	(—)	+	—	(—)	—	nd	+	—	—	+	+	+	+	—	+
Use as sole carbon source:															
Acetate	+	(+)	+	v	v	—	v	+	+	—	—	+	—	—	v
β-alanine	+	v	v	— <sup>δ</sup>	—	—	—	—	+ <sup>δ</sup>	—	—	—	—	—	(—)
DL-alanine	+	+ <sup>δ</sup>	+ <sup>δ</sup>	—	+ <sup>δ</sup>	— <sup>δ</sup>	+ <sup>δ</sup>	— <sup>δ</sup>	v	— <sup>δ</sup>	—	+	—	—	—
L-arginine	+	(+)	+	(—)	+	—	—	+	v	— <sup>δ</sup>	—	—	—	—	v
Glycine	+	(+)	v	— <sup>δ</sup>	+	—	—	—	+ <sup>δ</sup>	—	—	—	—	—	(—)
Inulin	+	(—)	—	—	—	—	—	—	—	—	—	—	—	—	(—)
L-lysine	+	(+)	+	—	—	—	v	—	v	—	—	—	—	—	(—)
Malonate	+	(—)	+ <sup>δ</sup>	—	—	—	v	— <sup>δ</sup>	v	—	—	—	—	—	—
L-phenylalanine	+	+ <sup>δ</sup>	—	—	v	— <sup>δ</sup>	+ <sup>δ</sup>	—	v	— <sup>δ</sup>	—	—	—	—	—
L-proline	+	+	+	(+)	+ <sup>δ</sup>	—	+ <sup>δ</sup>	+	+	— <sup>δ</sup>	—	—	—	—	(—)
Propanol	+	v	v	— <sup>δ</sup>	— <sup>δ</sup>	—	+ <sup>δ</sup>	—	+ <sup>δ</sup>	— <sup>δ</sup>	—	—	—	—	v
Pyruvate	+	+ <sup>δ</sup>	+	v	+ <sup>δ</sup>	—	+ <sup>δ</sup>	—	+	— <sup>δ</sup>	—	+	—	—	v
L-serine	+	(+)	—	— <sup>δ</sup>	+ <sup>δ</sup>	—	+ <sup>δ</sup>	—	+ <sup>δ</sup>	— <sup>δ</sup>	—	—	—	—	(—)
Succinate	+	v	+	v	+	—	+	—	—	—	—	+	—	—	v
L-tartrate	+ <sup>δ</sup>	(—)	— <sup>δ</sup>	— <sup>δ</sup>	— <sup>δ</sup>	—	— <sup>δ</sup>	— <sup>δ</sup>	— <sup>δ</sup>	— <sup>δ</sup>	— <sup>δ</sup>	— <sup>δ</sup>	— <sup>δ</sup>	— <sup>δ</sup>	(—)
L-tryptophan	+ <sup>δ</sup>	(—)	— <sup>δ</sup>	— <sup>δ</sup>	— <sup>δ</sup>	—	— <sup>δ</sup>	— <sup>δ</sup>	— <sup>δ</sup>	— <sup>δ</sup>	— <sup>δ</sup>	— <sup>δ</sup>	— <sup>δ</sup>	— <sup>δ</sup>	(—)
Uracil	+ <sup>δ</sup>	v	— <sup>δ</sup>	— <sup>δ</sup>	— <sup>δ</sup>	—	— <sup>δ</sup>	— <sup>δ</sup>	— <sup>δ</sup>	— <sup>δ</sup>	— <sup>δ</sup>	— <sup>δ</sup>	— <sup>δ</sup>	— <sup>δ</sup>	— <sup>δ</sup>
Sensitivity to:															
0/129 (150 µg)	—	v	nd	nd	+	nd	v	—	—	nd	+ <sup>δ</sup>	+ <sup>δ</sup>	+	+ <sup>δ</sup>	— <sup>δ</sup>
Tetracycline (30 µg) <sup>  </sup>	+	v	nd	nd	+	nd	+	+	+	nd	+	+	+	+	(+)

\*1. *A. johnsonii*; 2. *Alt. macleodii*; 3. *H. venusta*; 4. *M. flavimaris*; 5. *Pa. piscicida*; 6. *Pa. tetraodonis*; 7. *Pa. undina*; 8. *P. anguilliseptica*; 9. *P. mendocina*; 10. *R. galacensis*; 11. *S. japonica*; 12. *S. livingstonensis*; 13. *S. pacifica*; 14. *S. schelegeliana*; 15. *S. waksmanii*.

† All strains included in the same phena; ‡ strains included in different phena; <sup>||</sup> ungrouped strains; <sup>||</sup> one identified strain.

\*\* Number of identified strains of each species.

<sup>δ</sup> Useful discriminatory tests for rapid identification of each species selected after making a dichotomic differential table of species.

<sup>||</sup> No differential tests because all species gave the same or variable result.

Data are expressed as: nd: No data; +: Positive result (≥90% of positive results); —: Negative result (≤10% of positive results); (+): Mainly positive results (≥70% <90% of positive results); (—): Mainly negative results (≥10% <30% of positive results); v: Variable results (>30 <70% of positive results).

<sup>1</sup>, ADH: Thornley's arginine dehydrolase; KIA: Kligler iron agar; TCBS: thiosulphate citrate bile salt sucrose agar.

*M. flavimaris* were also isolated from phytoplankton. The genera *Alteromonas* and *Marinobacter* were also previously associated with phytoplankton by using molecular methods (Alavi et al. 2001, Hold et al. 2001, Seibold et al. 2001, Töbe et al. 2001, Green et al.

2004). The fact of identifying identical species on phytoplankton and oyster is probably a consequence of the process of filter feeding (Kueh & Chan 1985, Hariharan et al. 1995). Only *H. venusta* isolated from seawater and phytoplankton was excluded from oys-

ter, suggesting the inability of this species to colonize this organism (*Ostrea edulis*).

Although there are a limited number of strains isolated from each source, it seems that there is more diversity associated with the larval than with the reproductive stage of oyster when comparing the number of identified species and the total number of identified strains (11/16 and 5/12, respectively; Table 1). However, we cannot evaluate the diversity with the seed, seawater, or phytoplankton, because the number of identified species or the total number of strains is too low (Table 1).

It is difficult to judge how numerically abundant or ecologically significant the species identified actually are, because their representation may simply reflect their selective enrichment in culture and not their numerical abundance. However, these isolates have a representative value as culturable aerobic bacteria associated with the culture of oyster in the NW of Spain because of the high number of strains isolated in different geographical areas over

a long time period. The presence of various biotypes in *Alt. macleodii*, *M. flavimaris*, and *S. waksmanii* adds more phenotypical variability to each species (Farto et al. 1999, Farto et al. 2003, Montes et al. 2003), and this improves their phenotypical description.

A selection of phenotypical tests for rapid identification of potentially pathogenic or probiotic aerobic species was possible by the combination of both techniques. The improved and rapid identification of indigenous aerobic culturable bacteria could be extremely useful in industrial culture plants.

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## FIRST DETECTION OF AZASPIRACIDS IN MUSSELS IN NORTH WEST AFRICA

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**ABSTRACT** Outbreaks of lipophilic toxins have been recorded in the north Atlantic coast of Morocco since 1999, but are rare in the Mediterranean coast. Samples of mussels from the Atlantic coast where toxicity was detected by mouse bioassay were stored for further research. Chemical analysis by LC-MS conducted in mussels harvested from this region showed, in addition to the presence of okadaic acid (OA) and dinophysistoxin-2 (DTX2), the presence of azaspiracid-2 (AZA2) as the dominant form of the azaspiracid's (AZAs) family, followed by AZA1 (13% to 26%). AZA3 was rarely detected, and maximal concentrations found were between 3% and 8% of total AZA1/3. The presence of AZA2 and AZA1 was confirmed by mass spectra. Time series corresponding to the summer of 2004 and 2005 showed maximal concentration of AZAs appeared in July in both years. Correlation with occurrence of OA and DTX2, showed both toxin families could appear simultaneously in Moroccan mussels, but maximal concentrations found were always separated in time. This is the first report of azaspiracids in Morocco (NW Africa) and the first report outside of European coastlines.

**KEY WORDS:** azaspiracids, AZP, Morocco, LC-MS, DSP, mussels, *Mytilus galloprovincialis*

### INTRODUCTION

Azaspiracid poisoning (AZP) is a new human gastrointestinal illness discovered in 1995 after consumption of contaminated Irish mussels in the Netherlands (McMahon & Silke 1996). Azaspiracid-1 (AZA1) was the first molecule of the group to be structurally characterized (Satake et al. 1998). Now, at least more than 10 azaspiracid congeners are known (James et al. 2003b). From these, AZA1, AZA2, and AZA3 were the dominant compounds found in shellfish, followed by AZA4 and AZA5. The remaining ones (AZA6–11) were minor components. It is hypothesized so far that these lipophilic toxins accumulate in bivalve molluscs after feeding on the toxic microalgae *Prorocentrum crassipes*, previously considered to be toxicologically harmless (James et al. 2003a).

The symptoms of acute AZP intoxication include nausea, vomiting, severe diarrhea, and stomach cramps (Satake et al. 1998), which closely resemble the symptoms associated with diarrhetic shellfish poisoning (DSP). Toxicological studies showed that repeated administration of AZAs caused prolonged damage in the intestine and induced lung tumors in mice (Ito et al. 2002).

Although shellfish harvested in Ireland provoked the first incidents of human intoxications by azaspiracids, the research in other European countries has led to the discovery of these toxins in shellfish from the United Kingdom, Norway, France, and Spain (James et al. 2002a, Magdalena et al. 2003). Although AZP is a serious concern in Europe, its presence has never been reported outside European waters.

Outbreaks of lipophilic toxins have been recorded in shellfish from the north Atlantic coast of Morocco since 1999, but these toxins are rare in the Mediterranean coast (Taleb 2005). The Moroccan monitoring program to screen for the presence of lipophilic compounds employs the mouse bioassay (MBA), which does not allow distinguishing the contamination from different phycotoxin families, such as DSP or AZP, because these compounds are detected altogether. Although several methodologies exist to study the contamination with DSP, the only methodology available so far to confirm the specific presence of azaspiracids is liquid chroma-

tography coupled to mass spectrometry (LC-MS) (Ofuji et al. 1999). Samples of blue mussels collected in Morocco during the summers of 2004 and 2005 were screened by LC-MS for lipophilic compounds. Besides the presence of DSP toxins (to be reported elsewhere), the presence of azaspiracids was put into evidence and is now reported here for the first time.

### MATERIALS AND METHODS

#### Study Area

Samples of mussels (*Mytilus galloprovincialis*) were collected regularly from Oulad Ghanem and Dar Hamra at the Atlantic coast of Morocco (south of Casablanca, Fig. 1). Digestive glands (DG) were dissected from whole shellfish, homogenized, and extracted for MBA testing. The samples used for the study were selected from those collected in the period of summer outbreaks of samples positive to lipophilic toxins. Digestive glands of positive samples were stored frozen for ulterior analysis by LC-MS.

#### Sample Preparation and LC-MS Analysis

Digestive glands were homogenized, and a 5-g aliquot was taken and extracted by ultraturax with 20 mL aqueous 90% methanol in a screw-cap plastic centrifuge tube, followed by a 10 min centrifugation at 2500g. A 2-milliliter aliquot of the methanolic supernatant was transferred to 10-mL glass test tubes, washed with hexane (2 × 2 mL), and extracted with dichloromethane (2 × 2 mL). The combined dichloromethane layers were dried with anhydrous sodium sulphate, centrifuged, transferred to small glass test tubes, and dried at 38°C under reduced pressure on a RapidVap (Labconco, USA). After resuspension with 500-μL aqueous 90% methanol, contents were transferred to a 2 mL autosampler vial. Vortex mixing was used in all liquid-liquid and resuspension steps.

Analyses were performed on a LC-MS system from Hewlett-Packard 1100-Series, consisting of an in-line degasser, a quaternary pump, an autosampler, a column oven, and the 1946A single-quadrupole mass detector. High-purity nitrogen was obtained through an N<sub>2</sub>-Generator (Dominick-Hunter, Durham, England).

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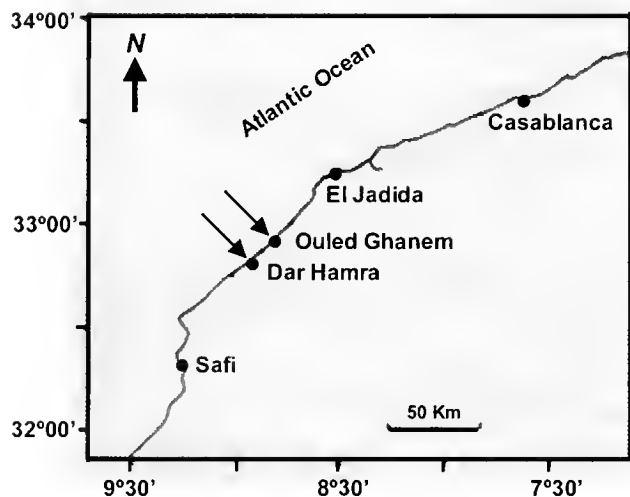


Figure 1. Localization of sampling sites.

A 1.0- $\mu$ L aliquot was separated for 12 min on a Merck *Lichrospher-100* RP-18 column (5  $\mu$ m, 125  $\times$  2 mm) protected by a guard column packed with the same material (4  $\times$  4 mm) using a mobile phase of acetonitrile-water (86:14, v/v), supplemented with 0.05% trifluoroacetic acid, and 50 mM formic acid pumped isocratically at 275  $\mu$ L/min. The eluent flow was diverted to waste for 2.0 min after sample injection, and MS detection was carried out from 2.0 min forward. Single ion monitoring (SIM) was used to record the signals from the  $[M + H]^+$  ions at:  $m/z$  828.5 (AZA3), 842.5 (AZA1), 844.5 (AZA4, AZA5), and 856.5 (AZA2). Mass spectra were obtained scanning in the range  $m/z$  600–900.

Dr. Phillip Hess and Dr. Terry McMahon, Marine Institute, Ireland, supplied a pure standard of AZA1 and mussels contaminated with AZA1–5.

## RESULTS

Analysis carried out in SIM mode for azaspiracids in mussel DG collected in the Atlantic coast of Morocco showed the presence in some samples of azaspiracid-2 (AZA2), followed by a minor contribution of AZA1. Only in samples where the concentration of AZA2 was highest, it was possible to detect also AZA3 (Fig. 2a). Analysis in SCAN mode of the most contaminated samples allowed the confirmation of AZA1 and AZA2 by mass spectra (Fig. 2b, c, respectively). AZA3 was not found in concentrations enough to allow confirmation by mass spectra. Although the presence of AZA4/5 was screened, no evidence of their presence was registered.

The temporal evolution of AZAs was studied in mussels collected in Oulad Ghanem in 2004 and 2005 (Fig. 1). In 2004, concentration of AZA2 and AZA1 was maximal in the beginning of July, then declined, and in autumn increased again (Fig. 3a). In 2005, the major peak of AZAs occurred again in mid July, and throughout the summer it remained fairly constant from 0.20  $\mu$ g/g down to 0.10  $\mu$ g/g (Fig. 3b). A similar evolution of toxic profiles was obtained with mussel samples collected in Dar Hamra in 2005 (data not shown). AZA2 was in all samples tested the dominant toxin (Fig. 3c, d). When detected, levels of AZA1 fluctuated between 13% and 26% (Figs. 3c, d). Only in samples from 2005 AZA3 was detected, and maximal concentrations found were between 3% and 8% (Fig. 3d).

The evolution of the summed concentrations of AZAs in mussels collected in Oulad Ghanem in 2004 and 2005 was compared with the concentration of DSP toxins found in the same samples, corresponding to the sum of hydrolyzed OA and DTX2 (data to be reported elsewhere). From the data presented in Figure 4, one observes that maximal concentrations of AZP toxins appear after the decline of DSP peaks (mid September 2004 and mid July 2005). Because data for the early summer of 2004 is incomplete, it is not possible to conclude if the AZA's peak from mid July

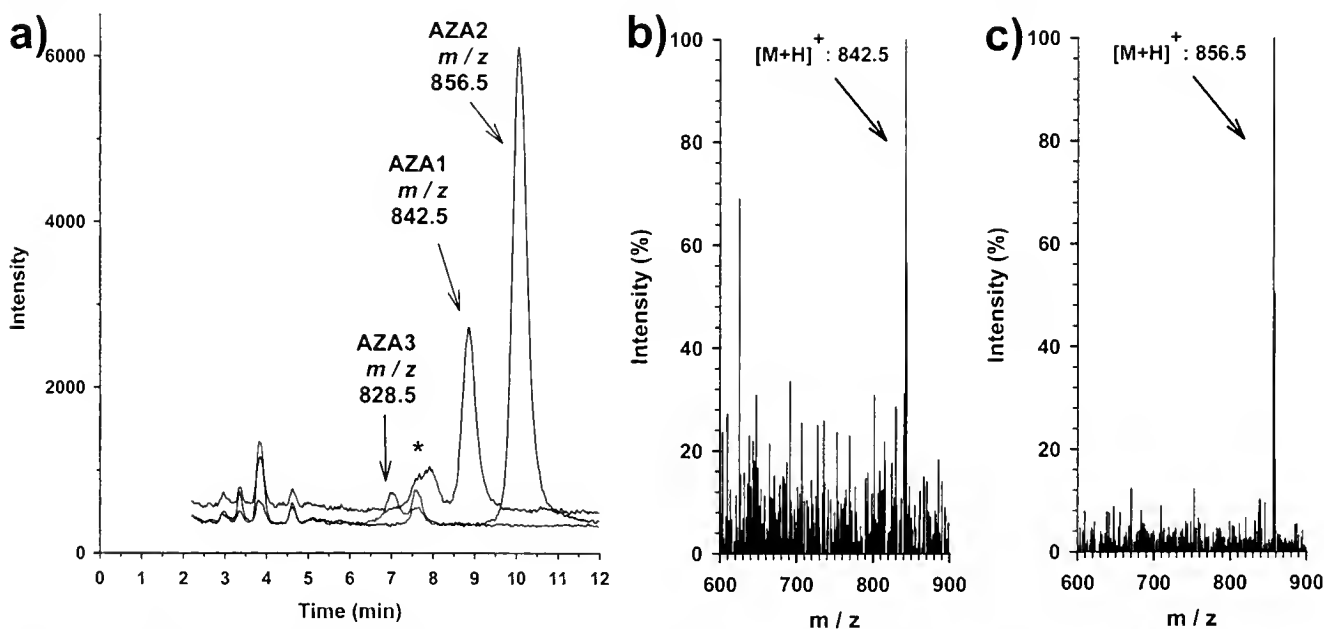


Figure 2. LC-MS analysis of mussel DG from Jemâa Ouled Ghanem collected on July 2005 (a) superposition of three SIM signals:  $m/z$  828.5 (corresponding to AZA3), 842.5 (AZA1), and 856.5 (AZA2); (b) and (c), mass spectra of AZA1 and AZA2, respectively, obtained from the same sample analyzed in SCAN mode.

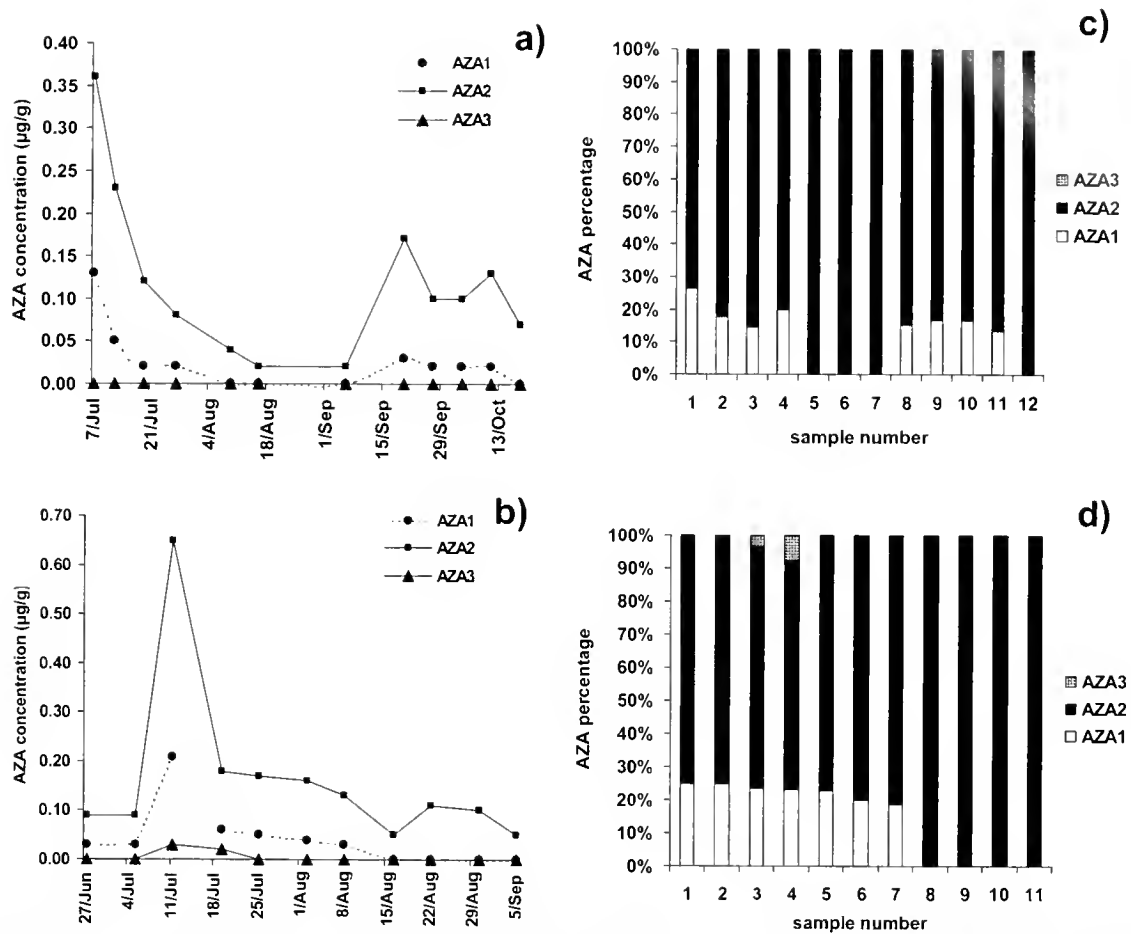


Figure 3. Evolution of AZAs concentration in mussel digestive glands collected in Jemâa Ouled Ghanem in (a) 2004, (b) 2005 and evolution of their respective percentages (c) and (d).

follows a declining DSP peak, or simply precedes a new DSP raising peak.

### DISCUSSION

This study reports for the first time the presence of azaspiracids in the NW African coast, and is the first time AZAs are reported

in shellfish from such low latitude (32°50'904N, 8°53'500W) and outside Europe. The presence of AZA2 in shellfish from the south coast of Portugal was previously suspected, but the low levels found of the suspect compounds did not allow their unambiguous confirmation, mainly because that study used whole flesh (Vale 2004). According to Hess et al. (2005), these toxins are on average

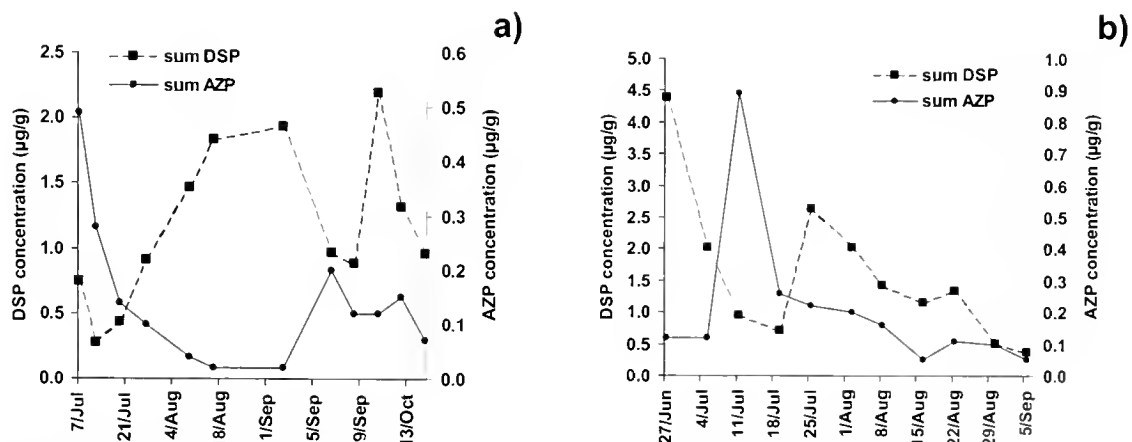


Figure 4. Comparison between concentration of DSP toxins and AZP toxins in mussel digestive glands collected in Jemâa Ouled Ghanem in (a) 2004, (b) 2005.

circa 5 times more concentrated in DG, compared with the whole mussel.

The simultaneous presence of DSP and AZP toxins may occur in Moroccan mussels, but maximal concentrations of these two distinct toxin families are separated in time. Evidence from Irish mussels points also that contamination with AZP may follow contamination with DSP (Clarke et al. 2006), but appearance of AZP before the rise in DSP toxins may also occur (Hess et al. 2002). Because the species hypothesized to produce AZAs is known to be heterotrophic, its growth might depend on the appearance of conditions that favor the blooming of certain algae species necessary for predation, amongst which also proliferate the DSP producers.

In the Atlantic Moroccan coast, the occurrence of AZP seems to be recurrent, and maximal levels may take place over the summer, although other seasons of the year were not studied yet for the presence of AZP. After the launching of the lipophilic toxin monitoring along the Moroccan coast by mouse bioassay (MBA), the recurrence of lipophilic toxin outbreaks in the Atlantic coast was recorded during the summer time almost every year since 1999,

but in the Mediterranean coast occurrence of this type of toxins is rare (Taleb, 2005). For the moment, at least two families of lipophilic compounds are implied in the observed mouse bioassay toxicity. So far, these are the most relevant families of toxins for public health, as other families, such as yessotoxins and pectenotoxins, do not seem to be relevant for human health because of their low oral potency (Anonymous 2004).

The toxin profiles found in European shellfish (hepatopancreas) and plankton samples have been dominated by AZA1 (James et al. 2002a, James et al. 2003a, Magdalena et al. 2003). However, AZA3 has been reported to be the most abundant toxin in meat samples with the hepatopancreas excised (James et al. 2002b). Although variations in toxin profile have been observed, the results reported here of AZA2 dominance might point to a difference in the phytoplankton-producing species. Indeed, the finding of AZAs at such low latitudes may raise the question if more than one species is involved in AZA production and also concerns of a more universal distribution of AZAs than previously believed.

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## MICROSCOPIC ANATOMY OF FOOT OF THE SPINY TOP SHELL, *BATILLUS CORNUTUS* (LIGHTFOOT, 1786) (GASTROPODA: TURBINIDAE)

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**ABSTRACT** Foot structures of the spiny top shell, *Batillus cornutus* were investigated by light and transmission electron microscopy. The foot was composed of epithelial layer, connective tissue layer, and muscular layer from outside inward. The epithelial layer was multiple-folded and simple and was composed mostly of columnar and mucous cells. Epithelial layer thickness was approximately 40  $\mu\text{m}$  in the pedal body and approximately 30  $\mu\text{m}$  in the opercular zone. Distribution of mucous cells in the epithelial layer was found to be higher in the opercular zone than those in the pedal body. Mucous cells contained acidic material abundant in sulfate and carboxylate group, from the results of AF-AB reaction. From TEM observation, the epithelial layer is composed of epithelial cell, ciliated cell, absorptive cell, and secretory cell. Secretory cells can be classified into six types (A, B, C, D, E, F) in accordance with the shapes and ultrastructural characteristics of secretory granule. Type A secretory cell is the most abundant cell of the six types of secretory cells. The type of muscle fibers was mostly smooth muscle fibers. Muscle fibers can be divided into two types, one that is composed only of thin microfilaments and the other composed of thin and thick microfilaments.

**KEY WORDS:** foot, microscopic anatomy, *Batillus cornutus*, TEM, top shell

### INTRODUCTION

The top shell, *Batillus*, belongs to Turbinidae of Gastropoda, and it is widely distributed throughout temperate coastal waters to tropical coastal waters including the Pacific, Indian, and Atlantic Ocean. Among these, spiny top shell, *Batillus cornutus* is a settled gastropod thriving in the waters of the southern part of the East Sea and coast of Cheju Island in Korea, southern coastal waters of Japan, and reef ridden coastal waters of the Yellow Sea of China. The spiny top shell is one of the most important food resources in Korea, along with abalone, placing it at a very important position industrially (Yoo 1988).

The foot of the gastropod is an organ with flexibility corresponding to the motile system, and it performs functions such as mobility, attachment, capturing of food, mating, spawning, and shell cleaning (Voltzow 1994). The foot is composed of muscular tissue and nerve bundles, and it is partially covered by an operculum (Bullock 1965).

Study on the pedal and muscular structure of the gastropod includes reports on *Patella vulgate* (Jones & Trueman 1970), *Neritina reclivata* and *Thais rustica* (Gainey 1976), *Patella vulgate* and *Acmaea tessulata* (Grenon & Walker 1978), *Bullia digitalis* (Trueman & Brown 1976, 1987), *Bullia rhodostoma* (da Silva & Hodgson 1987), patellid limpets (Frescura 1987; Frescura & Hodgson 1990a, 1990b), *Busycon contrarium* and *Haliotis kamtschaticana* (Voltzow 1990), and *Nassarius kraussianus* (Trueman & Hodgson 1990).

However, in relation to these studies, there is no biological study on the foot structure of the spiny top shell, *Batillus cornutus*. Therefore, this study aims to describe the ultrastructure of the foot of the spiny top shell and to provide basic information for future study on structural changes of the foot in accordance with environmental and physiological changes.

### MATERIALS AND METHODS

The spiny top shells were collected by divers in the coastal waters of Modori, Cheongsanmyeon, Wandogun, Cheollanamdo Korea (N 34°13', E 126°47') (Fig. 1) May 2003. Thirty spiny top

shells in shell height 60.0–69.9 mm were used in this study. The sampled individuals were measured, the shell removed, and the pedal tissue was fixed after dividing into the pedal body and opercular zone (Fig. 2).

For light microscopy the tissues were fixed in Bouin solution and then prepared according to the following paraffin methods: H-E double stain, Masson trichrome stain, PAS reaction, AB-PAS (pH 2.5) reaction, Alcian blue (pH 1.0) reaction, and AF-AB reaction were used for sections.

For transmission electron microscopy the specimens were fixed in 2.5% glutaraldehyde solution (pH 7.2 buffered in 0.1M phosphate buffer) for 2–4 h at 4°C and postfixed in 1% osmium tetroxide ( $\text{OsO}_4$ ) solution for 2 h at 4°C. After fixation the specimens were washed in 0.1 M phosphate buffer, dehydrated by ethanol step-by-step, and finally embedded in Epon 812. Ultrathin sections (70 nm in thickness) were put on copper grids (200-mesh), double stained with uranylacetate and lead citrate, and finally examined using a transmission electron microscope (JEM-1200EXII, JEOL).

Stain affinity of the mucous cell was determined by using the Pantone Formula Guide (Pantone Inc. USA) as standard, and its unique code was indicated in parenthesis. Image analyzer (IMT, Visus, USA) was used to quantify the histological characteristics.

### RESULTS

The foot of the spiny top shell is the organ that extends from the dorsal side of the anterior portion of the body and continues onto the head. Overall color of the foot was dark brown and the portion

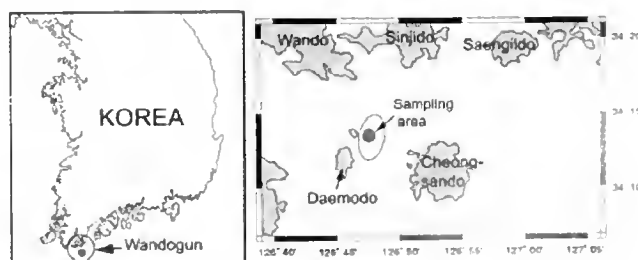


Figure 1. Sampling area of the spiny top shell, *Batillus cornutus*.

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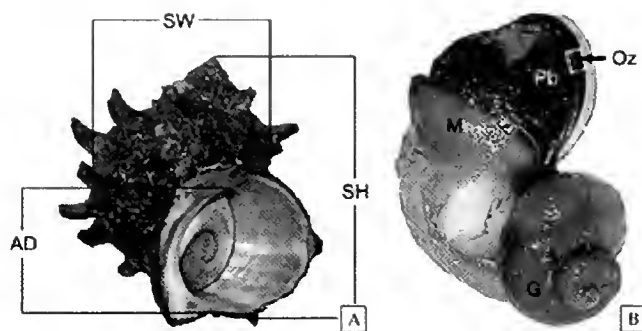


Figure 2. Morphology of the spiny top shell, *Batillus cornutus*. (A) Morphometric characteristics of the shell. AD, aperture diameter; SH, shell height; SW, shell width. (B) Region of the sampled tissue. G, gonad; M, mantle; Oz, opercular zone; Pb, pedal body.

attached to the operculum was a wrinkled light orange-colored muscular mass.

Histological structure of the foot was composed of an epithelial layer, connective tissue layer, and a muscular layer from outside inward. The epithelial layer was a simple multiple-folded layer composed mostly of columnar and mucous cells (Figs. 3, 4).

The epithelial layer was approximately 40  $\mu\text{m}$  thick in the pedal body and approximately 30  $\mu\text{m}$  in the opercular zone (Fig. 5). A well-developed striated border was formed on the free surface of the epithelial layer (Fig. 3B). Mucous cells were observed between columnar epithelial cells in the epithelial layer, and these cells exhibited vacuoles in H-E stain and Masson triple stain (Figs. 3A, B; 4A, B).

Mucous cells reacted weakly in PAS reaction in the pedal body (Fig. 3C) and opercular zone (Fig. 4C). However, mucous cells revealed a blue color (300 C) in AB-PAS (pH 2.5) reaction (Figs. 3D; 4D) and AF-AB reaction (pedal body: 2995 C; opercular zone: 306 C) (Figs. 3F; 4F). Distribution of mucous cells in the epithelial layer was found to be higher in the opercular zone than those in the pedal body (Fig. 6).

The connective tissue layer appeared very weak between the epithelial and muscular layer (Fig. 1B).

The muscular layer is composed of connective tissue and muscular fiber bundles with the presence of hemolymph sinus. Muscular fiber bundles were distributed regularly in horizontal and vertical directions, with higher density in the opercular zone than in the pedal body (Fig. 4B).

From TEM observation, the epithelial layer is composed of epithelial, ciliated, absorptive, and secretory cells.

Epithelial cells were columnar and approximately 30  $\mu\text{m}$  in height both body and opercular zone of the foot (Figs. 7A, C). In addition, microvilli of approximately 2  $\mu\text{m}$  height were developed commonly on the free surface of epithelial cells, and tight junctions of apical lateral aspect and membrane interdigitations were found between the epithelial cells (Fig. 7B). However, these columnar epithelial cells displayed differences in shapes and positions of the nucleus. In the columnar epithelial cell of the pedal body, the shape of the nucleus was irregular and oval-shaped and located in the middle or basal portion of the cell (Fig. 7A). In comparison, the nucleus of the columnar epithelial cell in the opercular zone had an elongated oval-shape with length equivalent to 2/3 of the cell length (Fig. 7C).

Ciliated cells were better developed in the epithelial layer of the body than those of opercular zone of the foot. These ciliated cells have well-developed cilia on the free surface. The length of these cells was approximately 45  $\mu\text{m}$  and the cilia length was approximately 4  $\mu\text{m}$ . These cells have a rectangular-shaped nucleus of approximately 17  $\mu\text{m}$  in the basal portion (Fig. 8A). Tubular mitochondria are clustered in the upper portion of the cytoplasm, and the ciliary rootlet is connected to mitochondria (Fig. 8B). Cross section of the cilia showed "9+2" microtubular structure of approximately 200 nm in diameter (Fig. 8C).

Cells with absorptive function that is rarely observed in the epithelial layer have microvilli on the free surface; and several pinocytotic vesicles, multivesicular bodies, and mitochondria were found in the upper portion of cytoplasm (Fig. 8D).

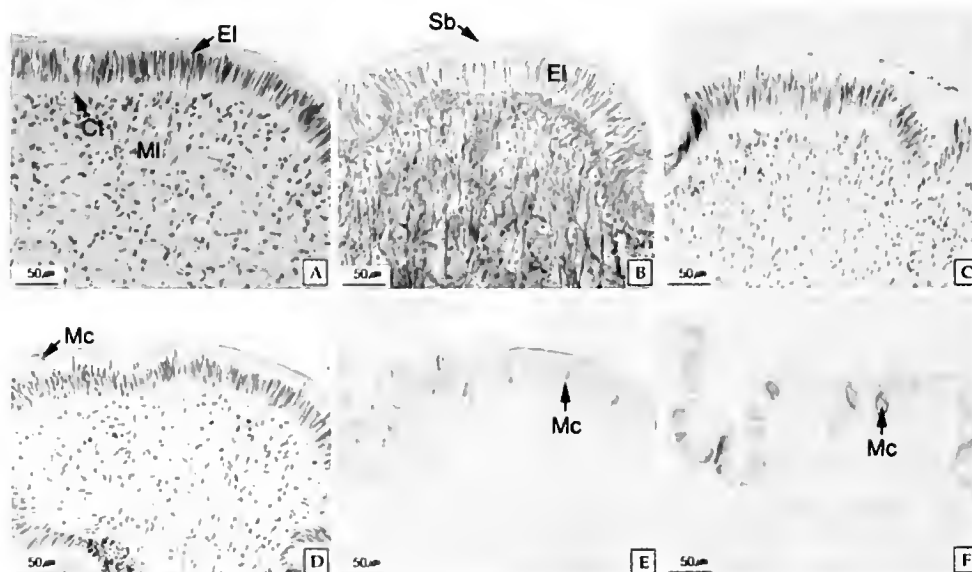


Figure 3. Cross section of the pedal body of the spiny top shell, *Batillus cornutus*. (A) Showing the epithelial layer (El), connective tissue (Ct) and muscular layer (Mi). H-E stain. (B) Masson's trichrome stain. (C) PAS reaction. (D) AB-PAS (pH 2.5) reaction. (E) Alcian blue (pH 1.0) reaction. (F) AF-AB reaction. Mc, mucous cell; Sb, striated border.

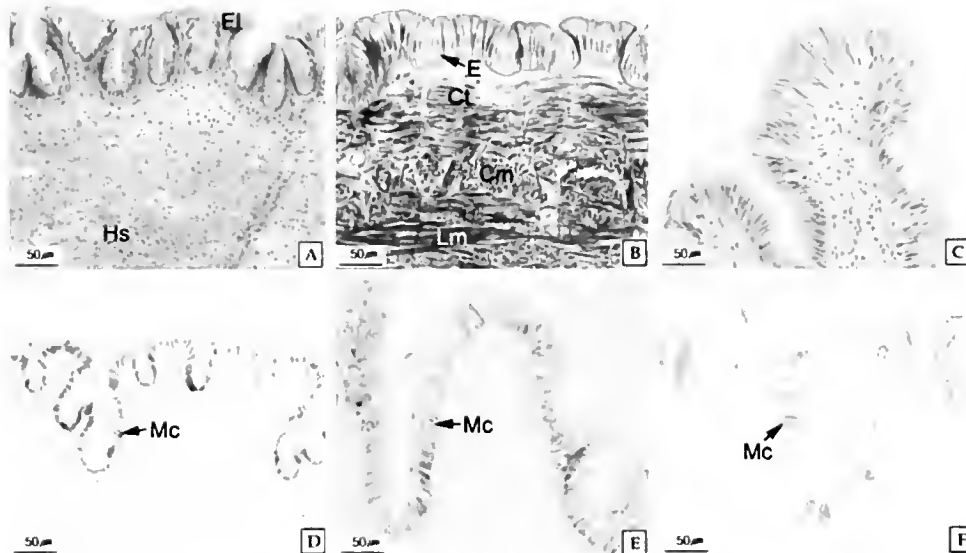


Figure 4. Cross section of the foot (opercular zone) of the spiny top shell, *Batillus cornutus*. (A) Section showing the hemolymph sinus (Hs) in the muscular layer. H to E stain. (B) Section showing the well-developed muscular layer. Masson trichrome stain. (C) PAS reaction. (D) AB-PAS (pH 2.5) reaction. (E) Alcian blue (pH 1.0) reaction. (F) AF-AB reaction. Cm, circular muscle; Ct, connective tissue; E, epithelium; El, epithelial layer; Lm, longitudinal muscle; Mc, mucous cell.

Secretory cells can be classified into 6 types (A, B, C, D, E, F) in accordance with the shapes and ultrastructural characteristics of secretory granule.

The type A secretory cell (Fig. 9A) is the most abundant cell of the six types of secretory cells. These cells have membrane-bounded secretory granules, and the granules had higher electron density in comparison with the secretory granules of other secretory cells (Fig. 9B). Furthermore, numerous rough endoplasmic reticula were found in the basal cytoplasm of these cells (Fig. 9A).

The type B secretory cell is circular. Electron density of the secretory granule was low, and the internal space of the secretory granule was filled with fine granular substances. The distribution of these cells was lower than the type A secretory cell but higher than all other secretory cells (Fig. 9C).

The type C secretory cell is clavate shaped. The nucleus of these cells was irregularly shaped and smooth endoplasmic reticula were scattered in the cytoplasm. Shape of the secretory granules was diverse, and the electron density was low (Fig. 9D).

The type D secretory cell is columnar with the nucleus located in the basal portion. Secretory granule of the cells did not have a

membrane, and the electron density was the lowest among the six types of secretory cells (Fig. 9E).

The type E secretory cell is circular. Rough endoplasmic reticula and secretory granules with diverse and lower electron density were scattered in the cytoplasm (Fig. 9F).

The type F secretory cell is oval-shaped. The distribution of these cells was the lowest among the six types of secretory cells. The secretory granule contained vacuoles with various electronic densities (Fig. 9G).

Numerous collagen fibers, fibrocytes, some muscle fibers, and hemocytes were observed in the connective tissue layer (Figs. 10A, B).

Some collagen fibers were observed in the muscular layer. In the longitudinal section, although the electron density was low, some transverse striations were distinguished in the collagen fibers (Fig. 10C).

Several types of muscle fibers were arrayed in various directions in the muscular layer, and they were mostly smooth muscle fibers. Muscle fibers can be divided into two types, one that is composed only of thin microfilaments and the other composed of

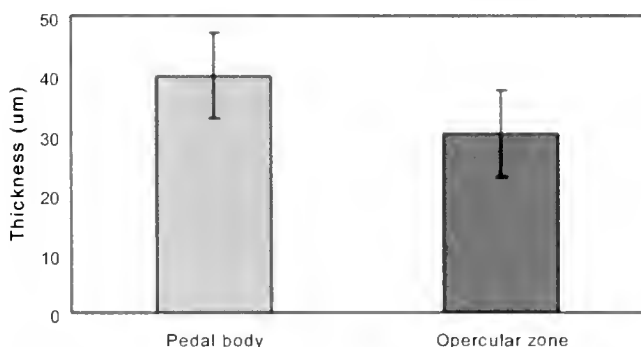


Figure 5. Epithelial layer thickness of the foot of the spiny top shell, *Batillus cornutus*.

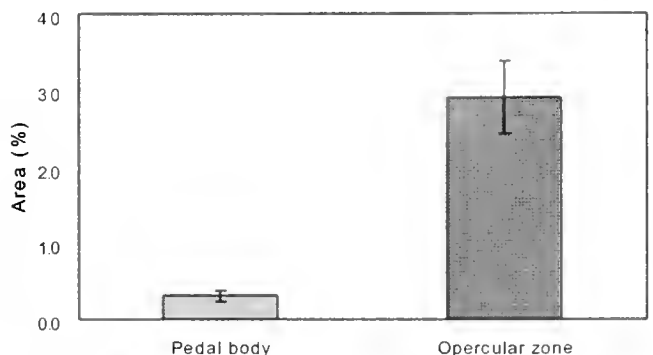


Figure 6. Mucous cell area of the foot of the spiny top shell, *Batillus cornutus*.

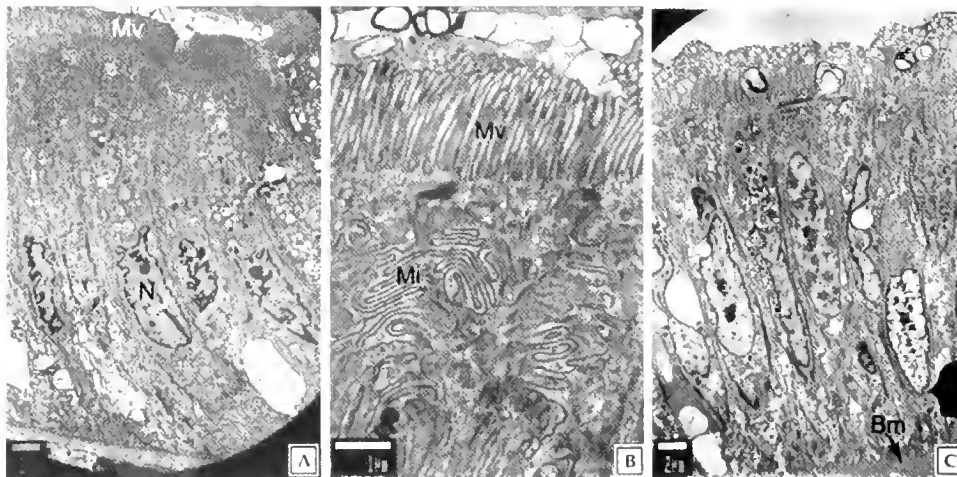


Figure 7. Ultrastructure of the pedal epithelial layer of the spiny top shell, *Batillus cornutus*. (A) Section showing the simple columnar epithelial layer,  $\times 2,000$ . (B) Microvilli (Mv) and intercellular junction of apical cytoplasm,  $\times 7,500$ . (C) Section showing the epithelial layer of opercular zone,  $\times 2,000$ . Bm, basal membrane; Mi, membrane interdigitations; N, nucleus.

thin and thick microfilaments. The former showed high-level distribution. The density of microfilament in the muscle fiber illustrated slight difference (Fig. 10D).

Muscle fibers composed of thin filament were categorized into two types. One type was composed only of muscle fibers with same electron density, and the other type was composed of muscle fibers with higher electron density in the cortex and lower electron density in the medulla (Fig. 10E). Thin filament muscle fibers were composed mainly of microfilaments approximately 20 nm in diameter. These have tubular mitochondria and a small number of sarcoplasmic reticula around the sarcolemma and some glycogen granules in the sarcoplasm (Fig. 10F).

In the muscle fiber in which thin and thick filament were mixed, the thin filament was approximately 10 nm in diameter, whereas that of the thick filament was 80 nm. Distribution of mitochondria and sarcoplasmic reticula in these muscle fibers was lower than the muscle fiber that was composed only of thin filament (Fig. 10G).

In addition, nerve cells were found between collagen fibers of the muscular layer (Fig. 10H).

## DISCUSSION

The foot of the gastropod is composed of the epithelial layer, connective tissue layer, and muscular layer. The epithelial layer is

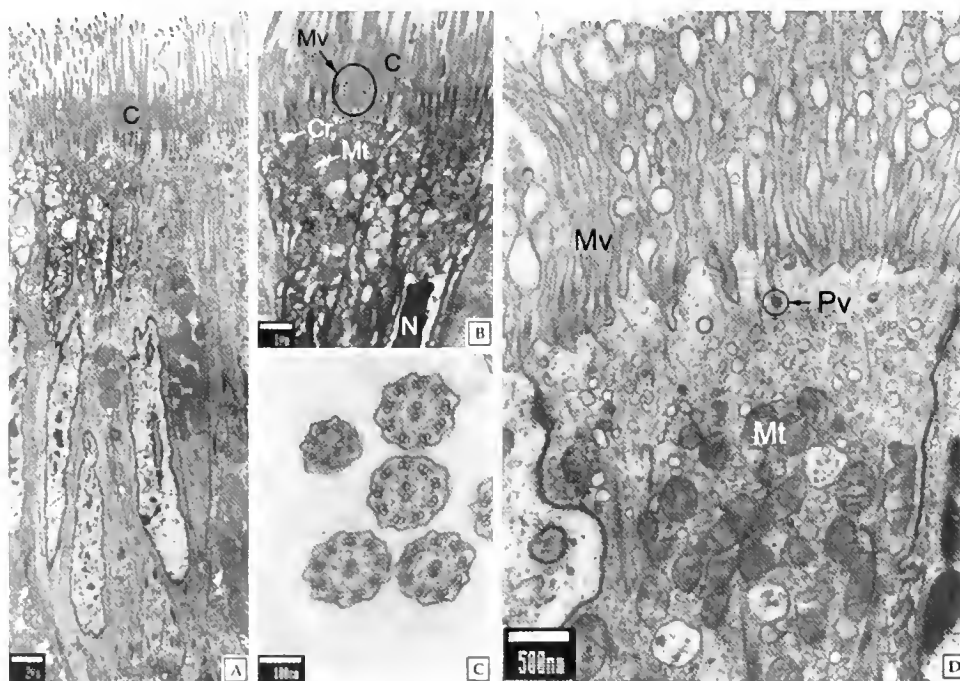


Figure 8. Ultrastructure of the pedal epithelial layer of the spiny top shell, *Batillus cornutus*. (A) Longitudinal section of ciliated columnar cell,  $\times 3,000$ . (B) Longitudinal section showing the numerous mitochondria (Mt) of cytoplasm in the ciliated cell,  $\times 6,000$ . (C) Cross section of cilia showing the “9+2” tubular system,  $\times 75,000$ . (D) Apical cytoplasm of pedal absorptive cell,  $\times 12,000$ . C, cilia; Cr, ciliary rootlet; Mv, microvilli; N, nucleus; Pv, pinocytotic vesicle.

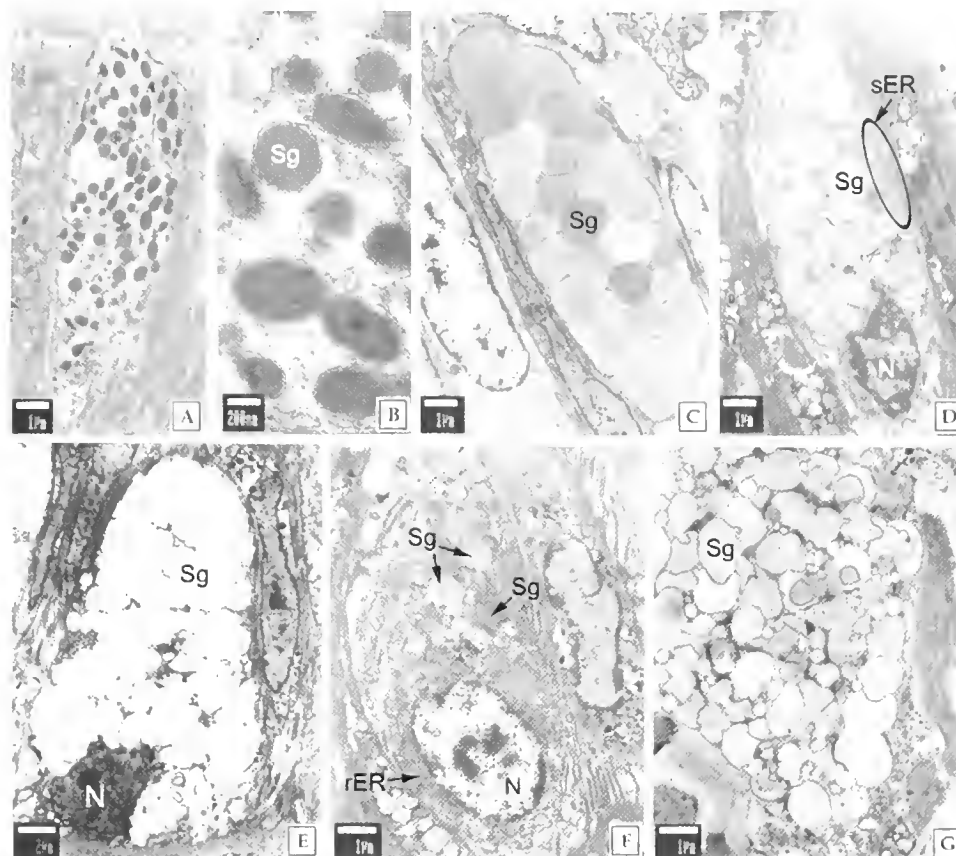


Figure 9. Ultrastructure of the pedal secretory cells of the spiny top shell, *Batillus cornutus*. (A) Type A secretory cell.  $\times 5,000$ . (B) Membrane-bounded secretory granules (Sg) of the type A secretory cell.  $\times 25,000$ . (C) Type B secretory cell.  $\times 5,000$ . (D) Type C secretory cell.  $\times 6,000$ . (E) Type D secretory cell.  $\times 3,000$ . (F) Type E secretory cell.  $\times 6,000$ . (G) Type F secretory cell.  $\times 6,000$ . N, nucleus; rER, rough endoplasmic reticula; sER, smooth endoplasmic reticula.

simple and composed of columnar epithelial and secretory cells. The connective tissue layer is relatively thin. In addition, the muscular layer is composed mainly of collagen fibers and smooth muscle fibers (Bullock 1965, Grenon & Walker 1978, da Silva & Hodgson 1987, Trueman & Hodgson 1990).

In this study, it was also found that the foot of the spiny top shell, *Batillus cornutus* was composed of epithelial layer, connective tissue layer, and muscular layer from exterior inward in cross section. The epithelial layer was simple and composed of columnar and mucous cells. Well-developed circular muscle, longitudinal muscle, and hemolymph sinus was observed in the muscular layer.

Generally, mucous cells in the epithelial layer of the mollusc foot contain acidic glycosaminoglycans within the sulfate and carboxylate group (Eble 2001). This study confirmed that the mucous cells contained acidic material abundant in the sulfate and carboxylate group from the results of AF-AB reaction.

Among gastropods, limpet, *Acmaca tessulata* had six types of secretory glands in the foot. The secretory substance is weakly acidic mucopolysaccharide and correlates with mobility function. *Patella vulgata* on the other hand has nine types of glands. Among these, six types are distributed in the foot, and contain weak acidic mucopolysaccharide and correlates with mobility function. The other three types are sole glands with highly viscous acidic mucopolysaccharide and attachment function (Grenon & Walker 1978).

In this study, 6 types (A, B, C, D, E, F) of secretory cells were

distinguished in the epithelial layer of the foot. Among these, C, D, and E type were observed mostly in the opercular zone, enabling one to predict that these cells are associated with operculum formation.

Although the muscle fiber of molluscs, in comparison with the vertebrates, is limited, transverse striations are found in some smooth muscle cell (Lowy & Vibert 1967; Millman & Bennett 1976; Sobieszek 1973). Characteristics of muscle fiber are diverse and contain paramyosin (Chantler 1983).

Types of muscle fiber composing the foot muscle of mollusc are categorized based on the arrangement of dense body, distribution of thick filament, ratio of thick microfilament and thin microfilament, invagination of sarcolemma, composition of sarcoplasmic reticula, and mitochondrial arrangement (Nicaise & Amselem 1983). Foot muscle of *Batillus rhodostoma* of Turbinidae illustrates intermediate type of muscle forms found in some reported molluscs (Lowy & Vibert 1967, Sobieszek 1973).

The foot muscle of *Nassarius kraussianus*, a gastropod, is composed mostly of smooth muscle fibers and collagen fibers. Although there were some muscle fibers with 4–9  $\mu\text{m}$  in diameter, the majority had 3–4  $\mu\text{m}$  in diameter, and has numerous membrane interdigitations between each cell. Muscle fiber is composed of thick filament with density of  $131 \pm 9.1 \mu\text{m}^{-2}$ ,  $40.6 \pm 4.1 \text{ nm}$  in diameter, and length of 7–10  $\mu\text{m}$ . Well-developed weakly acidic glycogen granules of 2  $\mu\text{m}$  in length and 0.5  $\mu\text{m}$  in diameter are found in the sarcoplasm (Trueman & Hodgson 1990).

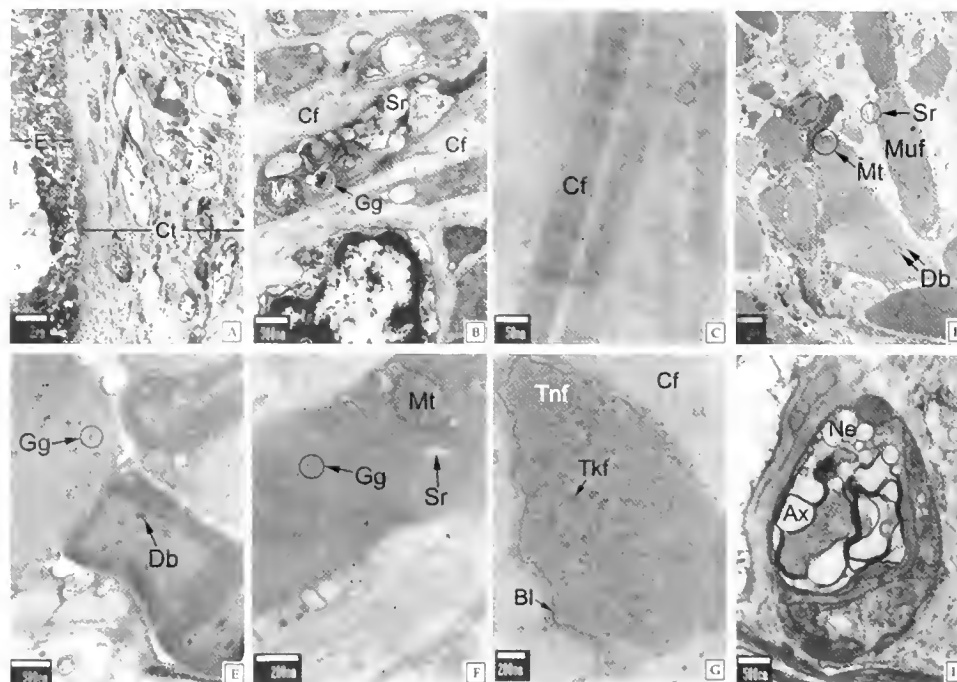


Figure 10. Ultrastructure of the pedal connective tissue and muscular layer of the spiny top shell, *Batillus cornutus*: (A) Section showing the epithelial layer (E) and connective tissue (Ct).  $\times 3,000$ . (B) Section showing the collagen fibers (Cf) and muscle fibers in the connective tissue layer.  $\times 12,000$ . (C) Longitudinal section of collagen fiber.  $\times 120,000$ . (D) Section showing the various muscle fibers (Muf) in the muscular layer.  $\times 2,500$ . (E) Cross section showing the two types of muscle fiber composed of thin filament.  $\times 15,000$ . (F) Longitudinal section of muscle fiber composed of thin filament showing the tubular mitochondria (Mt), sarcoplasmic reticula (Sr) and some glycogen granules (Gg).  $\times 25,000$ . (G) Cross section showing the muscle fiber of composed of thin filaments (Tnf) and thick filaments (Tkf)  $\times 40,000$ . (H) Nervous cell in the muscular layer.  $\times 12,000$ . Ax, axon; Bl, basal lamina; Db, dense body; Ne, nerve ending.

In the foot muscle of the whelk, *Bullia rhodostoma* of Nassariidae of Gastropoda, two types of muscle fibers, namely A and B types were distinguished. Among these, A-type is striated muscle fiber observed mainly in propodium, which enables fast shortening of the foot when crawling or burrowing. Dense body ( $94.70 \pm 7.50$  nm in diameter) of the A-type muscle fiber is arranged vertically along the long axis of the muscle fiber. Ratio of thin filament to the thick filament is more than 1:10 and the mitochondrial area is approximately 25%. The B-type is of smooth muscle fiber type that is mostly found in the metapodium, and functions to maintain greater tension. Dense body is rarely found in the B-type muscle fiber. The diameter of the dense body is  $84.40 \pm 2.47$  nm with irregular arrangement. Ratio of thin filament to the thick filament is approximately 1:12–1:30 with mitochondrial area at 35%. The mitochondrial distribution in the A-type muscle fiber is a dispersed pattern, whereas that in the B-type muscle fiber is clustered (da Silva & Hodgson 1987).

Frescura & Hodgson (1992) categorized the muscle fibers of columellar muscles of six species (*Bullia rhodostoma*, *Burnupena cincta*, *Halionis spadicea*, *Siphonaria capensis*, *S. concinna*, *Turbo sarmaticus*) belonging to Prosobranchia into Type I and Type II. Muscle fiber of Type I has small number of dense body, mito-

chondria and sarcoplasmic reticula. Thin and thick filaments are irregularly mixed in these muscle fibers. The diameter of thick filament measured in the preparation was 26 nm for *Turbo sarmaticus* and 69 nm for *Burnupena cincta*, illustrating differences depending on the species. In comparison, muscle fibers in the Type II category are composed of thin filament and have a striated appearance. These muscle fibers are connected with collagen fibers and exhibit difference in the development of cell organelles.

In this study, transverse striations were not clearly observed in the muscle fiber of the foot of the spiny top shell. Types of muscle fiber were distinguished into a type in which thin and thick filaments were present in mixture and the other type composed only of thin filament. It was determined that these two types corresponds to the "Type I" and "Type II" muscle fiber reported by Frescura & Hodgson (1990a, 1990b, 1992). Such result of this study and distribution of thick filament, invagination of sarcolemma and arrangement of dense body, and sarcoplasmic reticula and mitochondria illustrated the same features as previous reports. However, the finding that muscle fiber composed only of thin filament is categorized into two types is deemed to be a characteristic of muscle fiber of the spiny top shell, *Batillus cornutus*.

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## BIODIVERSITY OF SESSILE AND MOTILE MACROFAUNA ON INTERTIDAL OYSTER REEFS IN MOSQUITO LAGOON, FLORIDA

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**ABSTRACT** Our research focused on determining the diversity and abundance of sessile and motile macrofauna that use intertidal reefs of the eastern oyster *Crassostrea virginica* for feeding, settlement space or refuge in Mosquito Lagoon, Florida. Five replicate lift nets were deployed at six sites (three impacted reefs with seaward margins of disarticulated shells, three reference reefs without dead margins) to determine the species composition and numbers present on these reef types. All nets were deployed intertidally on backreef areas on living oyster reefs, just above mean low water. One and a half liters of live oysters and oyster shells were placed in each net (1m<sup>2</sup>) on deployment. Nets were surveyed for all fauna monthly for one year. Metrics used to evaluate habitat use were species richness (total number of different species found) and density (total number of organisms per net). Comparisons were also made between community assemblages found on the two different types of reefs in the area (with and without dead margins) and for sessile species, recruitment on living oysters versus disarticulated shells. Forty sessile and 64 motile species of macroorganisms were found utilizing the oyster reefs in Mosquito Lagoon. However, recruitment on live oysters was twice that on disarticulated shells. Significant temporal variations were documented. When the two reef types were compared, however, no differences were found.

**KEY WORDS:** oysters, *Crassostrea virginica*, habitat use, fishes, decapods, barnacles, invertebrates, lift nets

### INTRODUCTION

Human activities threaten the productivity, diversity, and survival of coastal resources, leading to a growing need to understand and manage all coastal zones (e.g., Jackson et al. 2001). The Indian River Lagoon system (IRL) on the east central Florida coast is one such place. This estuary extends 251 km, from Ponce de Leon Inlet to Jupiter Inlet. The Lagoon system is a series of three distinct, but connected, estuaries: the Indian River, the Banana River and Mosquito Lagoon. This lagoon system may contain the richest biota of any estuary in North America (Provancha et al. 1992). It supports over 3,000 animal and plant species, 50 of which are listed as threatened or endangered. Commercially important intertidal reefs of the eastern oyster *Crassostrea virginica* are common in this estuarine system.

Diversity is extremely high in the IRL because of its location within a zoogeographic transition zone (e.g., Walters et al. 2001, Smithsonian Institution 2006). Researchers have documented the substantial species diversity of many habitats and taxa in IRL waters: seagrass and its associated organisms (e.g., Virnstein et al. 1983, Dawes et al. 1995); finfish (Gilmore 1977, Gilmore 1995, Tremain & Adams 1995); elasmobranchs (Snelson & Williams 1981) and decapods (Smithsonian Institution 2006). To date, there have been no studies of the biodiversity on intertidal oyster reefs in the IRL.

Three-dimensional reef structures of *Crassostrea virginica* are created by years of successive settlement of larvae on adult shells (Dame 1996). Through its structural complexity, these ecosystem engineers create heterogeneity that is rare in marine systems dominated by soft-bottom habitats (e.g., Bartol et al. 1999, Micheli & Peterson 1999). Organisms use oyster reefs for many different reasons; mobile species may: (1) feed directly on live oysters, (2) use shell surfaces for spawning and (3) seek refuge from predation

within oyster clusters (e.g., Tolley & Volety 2005), whereas sessile species use oyster reefs for attachment space.

Previous studies on intertidal oyster reef biodiversity include: Wells 1961 (North Carolina), Dame 1979 (South Carolina), Bahr and Lanier 1981 (south Atlantic coast), Crabtree and Dean 1982 (South Carolina), Wenner et al. 1996 (South Carolina), Coen et al. 1999a (South Carolina), Posey et al. 1999 (North Carolina), O'Beirn et al. 2004 (Virginia) and Tolley et al. 2005 (Florida, Gulf of Mexico). In most of these studies, the primary focus was on motile species (fish and crustaceans). Our study adds to this database by investigating the recruitment of motile macrofauna on backreef regions of intertidal oyster reefs of *Crassostrea virginica* in the IRL system along the Atlantic Coast of Florida. In addition, this is the first study in Florida to quantify diversity and abundance of all sessile macrofauna on oyster reefs.

### METHODS

#### Study Site

All research was conducted in Mosquito Lagoon, within the boundaries of Canaveral National Seashore (28°90.68W; 80°82.06N) (Fig. 1). Except where dredged, the average depth of the Lagoon is less than 1 m and the current is primarily wind-driven (Walters et al. 2001). Annual salinity ranges between 18 and 45 ppt, depending on rainfall (Grizzle 1990, Walters et al. 2001).

Within a 5-y period (1998–2003), the number of recreational boat registrations within the counties that border Mosquito Lagoon increased by 43% (Wall et al. 2005). This increasing intensity of year-round boating has helped create piles of disarticulated shells (dead margins) on the seaward edges of oyster reefs along major navigational channels in these shallow waters (Grizzle et al. 2002, Wall et al. 2005). We compared back-reef areas on reefs with and without dead margins to determine if recreational boating pressures influenced biodiversity.

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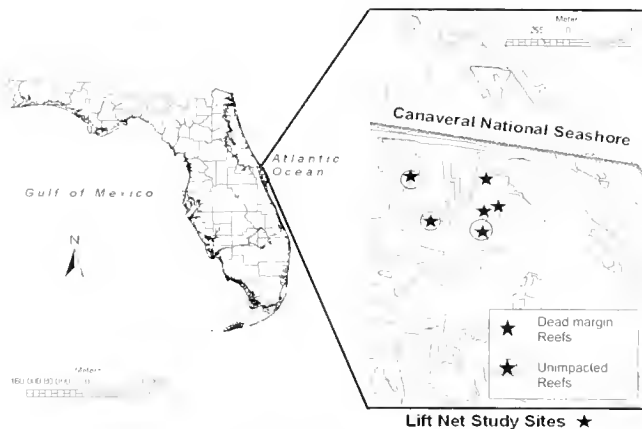


Figure 1. Lift nets study sites in Mosquito Lagoon, Florida.

#### Lift Net Field Sampling

Six oyster reefs were selected for this study, three impacted reefs (with dead margins) and three reference reefs (without dead margins). All were within a 5-km radius (Fig. 1). Five replicate lift nets were placed on the back-reef area of each reef. The protected back-reef areas were chosen to minimize the loss of nets caused by water motion.

Lift net methods were adapted from Crabtree and Dean (1982), Coen et al. (1996a), and later modified by Tolley et al. (2005) for use in Florida systems. We further modified the protocol to include the enumeration of sessile species recruiting to oyster reefs. Lift net frames were 1 m<sup>2</sup> and created from 3.8 cm diameter PVC. The nets were 0.5 m deep. The sides of the nets were made from 3.2 cm diameter opening mesh and the bottom was made from a 1-m square of 0.2-cm diameter opening mesh. The two mesh sizes were machine-sewn together using extra strength cloth thread. The sewn mesh was attached to the PVC frame with cable ties (tensile strength: 11 kg).

Lift nets were deployed intertidally, just above mean low water, on living oyster reefs. Volume normalized oysters and oyster shells in good condition (1.5 L) were placed in the lift nets. Half (0.75 L) were single, disarticulated shells from adults (Mean  $\pm$  SE length: 77.5  $\pm$  1.4 mm; weight: 21.5  $\pm$  1.1 g) and half were similar-sized live clusters collected from the oyster reef. All were mechanically scraped clean of epiflora and epifauna. New shells and clusters were placed into the nets each month. Additionally, at the time of net retrieval, all nets were cleaned to remove organisms that had settled on the mesh or PVC frames.

Lift nets were retrieved by swiftly picking up the nets on two sides and collecting all recruited motile and sessile organisms. In the laboratory, we identified all organisms within 24 h and returned them alive to Mosquito Lagoon. Only sessile organisms attached to oyster shells within the lift nets were counted. Nets were collected monthly for 12 mo (June 2004 to July 2005). No data was collected for September 2004 because Hurricanes Charley, Jeanne and Frances required removal of nets and prevented data collection. Specimens of each species were preserved in 70% isopropanol to create a species archive for the University of Central Florida.

#### Environmental Variables

Permanent temperature monitors (Onset Stowaway Tidbit Temperature Loggers) were attached to cinder blocks and de-

ployed at each site in water at the same depth as the lift nets. Temperature data were collected once each hour. Salinity was measured on net retrieval using a portable refractometer. Three sediment traps were deployed at each site at the same depth as the lift nets to determine sediment load accumulations during the 4-wk intervals between sampling. Each replicate, cylindrical PVC pipe sediment trap (10-cm diameter  $\times$  25 cm deep) was submerged flush with the substrate (Lenihan 1999). We capped traps underwater at the time of retrieval. The sediment traps were retrieved concurrently with the lift nets and new traps were immediately deployed to replace them. Total sediment mass was determined by drying samples at 60°C for 48 h in a drying oven (Econotherm Model Number 51,221,126) and weighing contents on a top-loading balance (O'Haus Scout 2-Model Number SC6010). Relative grain size was determined by grinding the dried sediment and sorting samples with a sieve (0.062 mm) to separate the silt/clay from the sand/gravel fractions.

#### Analyses

For all cases where analysis of variance (ANOVA) tests were run, prior to running the ANOVAs, homogeneity of variance and normality were tested using Levene and Kolmogorov-Smirnov tests. If significant differences were found with ANOVA, *post-hoc* Tukey-Kramer tests were run. Data assumptions of variance and normality were met for all ANOVAs at the  $P = 0.05$  level, thus the data were not transformed.

#### Sessile Macrofauna

Response variables of species richness (total number of different species) and density (total number of individuals) were analyzed using a 4-way, nested, ANOVA. The factors in the nested ANOVAs were: (1) reef type (reefs with dead margins or reference reefs), (2) month, (3) site and (4) shell type (disarticulated shells or live oysters in clusters). Reef type, month and shell type were fixed factors, whereas site was random. Shell type was nested within site, and site was nested within reef type.

#### Motile Macrofauna

Community metrics of motile species were similarly examined with a 3-way ANOVA. Response variables of species richness and density were examined as in sessile species. For each ANOVA, the factors were reef type (fixed), site nested within reef type (random), and month (fixed).

#### Sediment Loads

A 3-way ANOVA was conducted to test whether sediment loads on oyster reefs varied as a function of the following fixed factors: reef type (reference or dead margins) and month. The third factor, site, was random and nested within reef type.

## RESULTS

#### Biodiversity and Composition

#### Sessile Macrofauna

Twenty-five species of sessile invertebrates recruited to oysters and oyster shells in the lift nets during our study (Table 1). Barnacles in the genus *Balanus* (Arthropoda) dominated all samples numerically. Tube worms in the genus *Hydroides*, the jingle shell *Anomia simplex*, the eastern slipper shell *Crepidula astrasolea*,

TABLE 1.  
Total numbers of sessile species collected in lift nets on intertidal oyster reefs in Mosquito Lagoon, Florida.

Phylum	Species	Common Name	Total	6/04	7/04	8/04	10/04	11/04	12/04	1/05	2/05	3/05	4/05	5/05	6/05
Porifera	<i>Hymeniacidon heliophila</i>	Sun sponge	28	2	2	1	0	11	0	3	0	1	2	4	2
	<i>Halichondria melandocia</i>	Black volcano sponge	66	1	0	0	0	0	5	0	0	0	0	3	57
	<i>Cliona</i> spp.	Boring sponge	13	0	1	0	0	0	0	9	1	2	0	0	0
Cnidaria	<i>Aiptasia pallida</i>	Sea anemone	2	0	0	0	0	0	0	2	0	0	0	0	0
	<i>Haliplanella luciae</i>	Striped anemone	2	0	0	0	0	0	0	0	0	1	1	0	0
Annelida	<i>Hydroids</i> spp.	Tube worms	2842	658	491	760	250	134	111	66	33	65	55	42	177
	<i>Sabella</i> spp.	Feather duster worm	46	2	1	9	6	4	6	7	0	0	1	9	1
Arthropoda	<i>Balanus crenatus</i>	Ivory barnacle	8782	2070	2447	1380	720	430	210	145	87	132	99	251	811
	<i>Balanus amphitrite</i>	Purple striped barnacle	1524	438	461	450	46	16	13	9	1	5	6	8	71
Mollusca	<i>Crassostrea virginica</i> recruits	Eastern oyster	722	173	92	54	69	104	91	7	3	18	4	12	95
	<i>Anomia simplex</i>	Jingle shell	1120	186	178	184	151	93	83	41	4	43	45	80	32
	<i>Crepidula astrasolea</i>	Eastern slipper shell	1178	287	154	207	149	69	59	31	4	25	44	73	76
	<i>Crepidula fornicata</i>	Atlantic slipper shell	40	12	0	3	4	4	0	9	0	1	1	0	6
	<i>Diadora caryensis</i>	Keyhole limpet	3	1	1	1	0	0	0	0	0	0	0	0	00
	<i>Atrina rigida</i>	Pen shell	1	0	0	0	0	0	1	0	0	0	0	0	0
	<i>Tagelus divisus</i>	Jackknife clam	1	0	0	0	0	0	0	0	1	0	0	0	0
	<i>Brachidometes exustus</i>	Scorched mussel	4	0	0	2	0	0	1	0	1	0	0	0	0
	<i>Genkensis demissa</i>	Ribbed mussel	128	21	20	19	7	6	4	7	0	2	4	19	18
	<i>Mytella charruana</i>	Charru mussel	3	0	0	0	3	0	0	0	0	0	0	0	0
	<i>Lithophaga bisulcata</i>	Mahogany date mussel	1	0	0	0	0	0	0	0	0	0	0	0	1
Ectoprocta	<i>Bugula neritima</i>	Common bryozoan	195	1	1	0	0	2	0	16	0	18	82	48	27
	<i>Hippopurina verrilli</i>	Lacy bryozoan	40	0	0	1	28	11	0	0	0	0	0	0	0
	<i>Zoobotryon verticillatum</i>	Spaghetti bryozoan	2	0	0	0	0	0	0	0	0	0	0	0	2
	<i>Perophora viridis</i>	Encrusting ascidian	16	0	2	0	1	5	3	3	0	2	0	0	0
Chordata	<i>Styela plicata</i>	Rough sea squirt	87	0	0	0	26	17	2	1	0	1	15	6	19

and the eastern oyster *Crassostrea virginica* were also very abundant (Table 1). Mollusca represented the most abundant phyla, with nine species found. Other phyla represented included Annelida, Cnidaria, Porifera, Ectoprocta and Chordata (Table 1). Outside of the lift nets, 15 additional species of sessile organisms were found in small numbers on the intertidal oyster reefs and nearby subtidal areas throughout the course of our study in Mosquito Lagoon, although they do not represent any additional phyla (Table 2).

Measures of oyster community metrics with sessile invertebrates exhibited clear trends in Mosquito Lagoon. Species richness and the density differed temporally, because of the month of sampling (ANOVA:  $P < 0.001$ ; Fig. 2, 3; Table 3, Table 4). Richness was significantly higher during June, July, August and October ( $P < 0.001$ ; Fig. 2). Additionally, February had the lowest richness (Fig. 2). Density, the number of organisms per net, was significantly higher in June, July and August of 2004 than all other sampling periods (ANOVA:  $P < 0.001$ ; Fig. 3). Furthermore, spe-

cies richness and density were higher on living oysters in clusters than on single disarticulated oyster shells (ANOVA for both:  $P < 0.001$ ; Tables 3, 4; Fig 4). Species found only on live oysters included mussel *Lithophaga bisulcata*, ascidian *Perophora viridis* and bryozoan *Hippopurina verrilli*. Reef type (reefs with dead margins or reference reefs) did not have a significant influence on the community metrics, species richness ( $P = 0.098$ ) or density ( $P = 0.207$ ) (Tables 3, 4). Site did not have a significant effect on species richness (ANOVA:  $P = 0.964$ ) or density ( $P = 0.644$ ) (Tables 3, 4).

#### Motile Macrofauna

During this study, 64 motile species were found on oyster reefs in Mosquito Lagoon. Fifty-one species were collected using lift nets (Table 5) and an additional 13 species were observed by researchers elsewhere on reefs and in nearby subtidal waters (Table 2). Chordata was the most abundant phyla found to be

TABLE 2.

Additional macrofauna observed on oyster reefs within Mosquito Lagoon. These species were not collected in the lift nets.

Sessile Species		
Phylum	Species Name	Common Name
Annelida	<i>Polydora websteri</i>	Oyster mud worm
Mollusca	<i>Modiolus americanus</i>	Tulip mussel
	<i>Mercenaria mercenaria</i>	Hard shelled clam
	<i>Anadara transversa</i>	Transverse ark
	<i>Anadara ovalis</i>	Blood ark
	<i>Martesia cuneiformis</i>	Striated wood paddock
	<i>Crepidula convexa</i>	Convex slipper shell
Ectoprocta	<i>Conopeum</i> spp.	Lacy crust bryozoan
	<i>Zoobotryon verticillatum</i>	Moss bryozoan
	<i>Hippoporella verrilli</i>	
Chordata	<i>Mogula manhattensis</i>	Sea grape
	<i>Botryllodes nigrum</i>	Black tunicate
	<i>Botryllus planus</i>	Royal tunicate
	<i>Botryllodes schlosseri</i>	Goldenstar tunicate
	<i>Didemnum</i> sp.	
Motile species		
Phylum	Species Name	Common Name
Arthropoda	<i>Hexapanopeus angustifrons</i>	Narrow mud crab
	<i>Limulus polyphemus</i>	Horseshoe crab
	<i>Neopanope sayi</i>	Say's mud crab
	<i>Pinnotheres ostreum</i>	Oyster pea crab
Mollusca	<i>Aplysia brasiliana</i>	Sooty sea hare
	<i>Busycon contrarium</i>	Lightening whelk
	<i>Busycon spiratum</i>	Pear whelk
	<i>Fasciolaria hunteria</i>	Banded tulip
	<i>Fasciolaria tulipa</i>	True tulip
	<i>Melongena corona</i>	Crown conch
	<i>Pleuroploca gigantea</i>	Florida horse conch
	<i>Polinices duplicatus</i>	Atlantic moon snail
Chordata	<i>Symphurus plaginusa</i>	Blackcheek tonguefish

utilizing the oyster reefs, with 23 fish species found. Mollusca were the second most prevalent phyla, with 20 different species found (Tables 2, 5). Other phyla that were represented in the collections included: Arthropoda (18 species), Echinodermata (2 species) and Annelida (1 species) (Tables 2, 5). The bigclaw snapping shrimp *Alpheus heterochaelis* and the flat mud crab *Eurypanopeus depressus*, dominated the collections numerically year-round (Table 5).

Species richness and density differed because of the month of

TABLE 3.

Four-factor nested ANOVA comparing species richness of sessile organisms in lift nets. Factors were reef type (dead margin or reference; fixed), shell type (live clusters or disarticulated shells) nested within site nested within reef type (random), and month (fixed).

Source	df	Mean Square	F	Significance
Reef type	1	64.201	4.625	0.098
Site (Reef type)	4	13.881	0.133	0.964
Shell type (Site (Reef type))	6	104.321	42.236	<0.001
Month	11	85.401	34.576	<0.001
Residual	697			

TABLE 4.

Four-factor nested ANOVA comparing density of sessile organisms in lift nets. Factors were reef type (dead margin or reference; fixed), shell type (live clusters or disarticulated shells) nested within site nested within reef type (random), and month (fixed).

Source	df	Mean Square	F	Significance
Reef type	1	68406.006	2.265	0.207
Site (Reef type)	4	30195.728	0.657	0.644
Shell type (Site (Reef type))	6	45976.603	16.335	<0.001
Month	11	35020.837	12.443	<0.001
Residual	697			

sampling (ANOVA: both  $P < 0.001$ ; Table 6, Table 7). Richness was higher in November-December, January and May (Fig. 2). Density was significantly higher in June, November and December 2004 than any of the other sampling dates (Fig. 3). Reef type (reefs with dead margins or reference reefs) did not have a significant influence on species richness (ANOVA:  $P = 0.985$ ) or density ( $P = 0.624$ ) (Tables 6, 7). Site did not significantly affect species richness (ANOVA:  $P = 0.181$ ), however it did significantly affect density ( $P = 0.002$ ) (Tables 6, 7).

#### Environmental Variables

During the 13-mo study, the monthly mean temperatures in Mosquito Lagoon ranged from 16°C to 31°C (Fig. 5a). Salinity ranged from 25–35 ppt (Fig. 5b), falling within the typical average range of 25–45 ppt for the monthly mean in Mosquito Lagoon (Walters et al. 2001). The lowest salinity (25 ppt) occurred immediately after the 2004 hurricane season (Fig. 5b). Total sediment loads differed significantly between sites (ANOVA:  $P = 0.011$ ), but not reef type ( $P = 0.234$ ) (Table 8; Fig. 5c). After sediment loads were separated into fractions, percent silt/clay still did not differ significantly between reef type (ANOVA:  $P = 0.454$ ) or sites ( $P = 0.482$ ) (Table 9; Fig. 5d). During the months of June 2004 and June 2005, both sediment load and percent silt/clay differed temporally (ANOVA:  $P = 0.004$  and  $<0.001$ , respectively). Tukey results showed sediment loads to peak in June 2004,

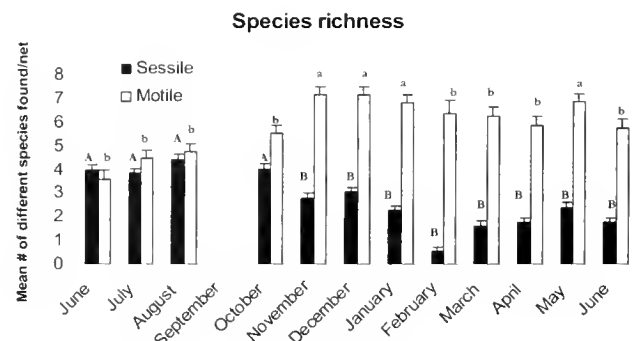


Figure 2. Monthly mean richness (total number of species)  $\pm$  SE per net per month from June 2004 to June 2005. September data are missing because of 2004 hurricane activity. When compared with ANOVA and a Tukey-Kramer *post-hoc* test, significantly higher months are depicted by A versus B for sessile species and a versus b for motile species.

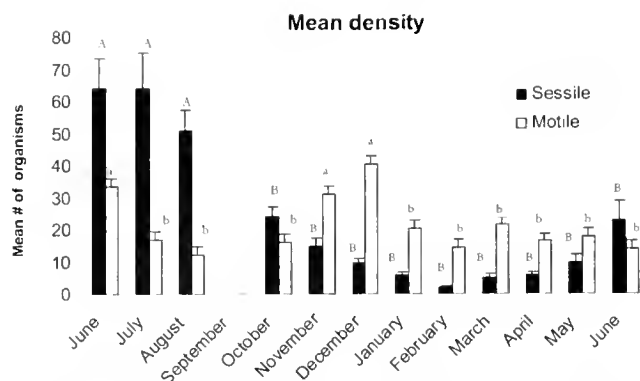


Figure 3. Monthly mean density (number of individuals)  $\pm$ SE per month from June 2004 to June 2005. September data missing because of 2004 hurricane activity. When compared with ANOVA and a Tukey-Kramer *post-hoc* test, significant higher months are depicted by A versus B for sessile species and a versus b for motile species.

whereas percent silt/clay fractions were highest in June and July 2004, and January 2005 (Fig. 5c, 5d).

## DISCUSSION

The assemblage of macrofauna associated with the intertidal oyster reefs during our lift net study was similar to those previously reported on oyster reefs in the southeastern United States (sessile species: Wells 1961; motile species: Meyer 1994, Breitburg 1999, Coen et al. 1999, Posey et al. 1999, Glancy et al. 2003,

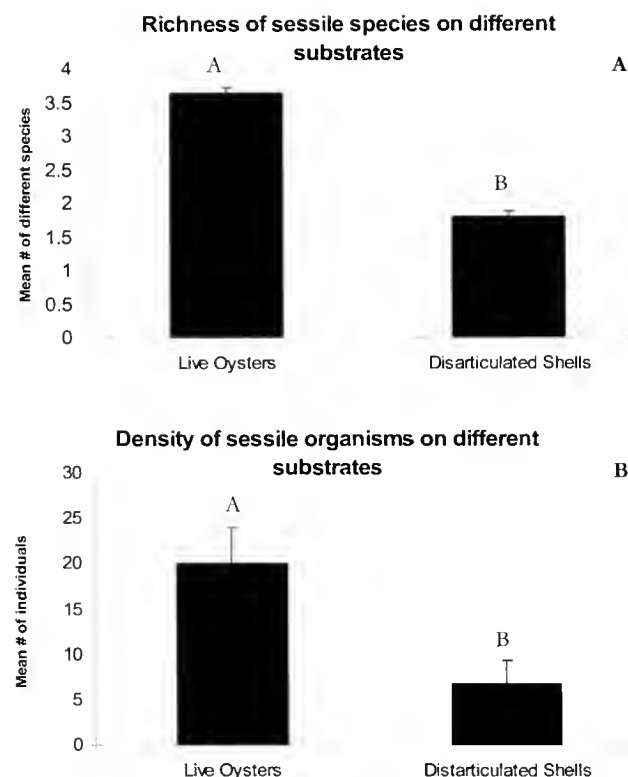


Figure 4. (a) Mean richness ( $\pm$ SE) of sessile organisms on live oysters compared with disarticulated shells. (b) Mean density ( $\pm$ SE) of sessile organisms on live oysters compared with disarticulated shells. Significant differences are depicted by A versus B.

TABLE 6.

Three-factor nested ANOVA comparing species richness of motile species in lift nets. Factors were reef type (dead margin or reference; fixed), site nested within reef type (random), and month (fixed).

Source	df	Mean Square	F	Significance
Reef type	1	0.003	0.000	0.985
Site (Reef type)	4	8.019	1.574	0.181
Month	11	37.699	9.340	<0.001
Residual	343			

Tolley et al. 2005, Tolley & Votely 2005). Our data also support earlier research conducted in the Indian River Lagoon system that looked at the sessile species diversity on hard substrata, although not specifically associated with *Crassostrea virginica* (Mook 1976, 1980, 1981 and 1983). As is typical for shellfish assemblages (O'Beirn et al. 2004), the oyster reef community within Mosquito Lagoon was dominated (in terms of abundance) by only a few taxa (i.e., Annelida, Arthropoda and Chordata) (Table 1 and 5).

## Sessile Macrofauna

The most abundant sessile species in the nets were in the genus *Balanus*. These organisms were present year-round on oyster reefs and numerically dominant in all nets. *Balanus eburneus*, the native ivory barnacle recruited to shells placed in lift nets during each month of our survey (Table 1). Monthly recruitment ranged from 87 recruits in February 2005 to 2,447 in July 2004. In fact, every net always had at least one *B. eburneus*. *Balanus amphitrite*, the purple striped barnacle, invaded the IRL approximately 100 y ago (J. Carlton pers. comm.). It was common but not as abundant as its congener (Table 1). Numbers of *B. amphitrite* decreased dramatically during the colder months of the year (Table 1). The abundance of these barnacles in this system outcompeting *C. virginica* for space may be associated with declines in oyster populations in Mosquito Lagoon (Boudreaux 2005). Dense sets of *Balanus* spp. monopolizing all free space on oyster reefs suggest intense spatial competition between oysters and barnacles in the IRL during summer and fall months (Boudreaux 2005).

The nonnative bivalve mussel, *Mytilus charruana*, was found during this study (Boudreaux & Walters 2006). This South American bivalve was first found in lift nets in August 2004 and has since rapidly spread within northern Mosquito Lagoon (Boudreaux & Walters 2006). Although low numbers of this species may have predated our study, no individuals were recorded in a 3-y study in these waters between 1998 to 2001 (L. Walters unpublished data).

TABLE 7.

Three-factor nested ANOVA comparing density of motile species in lift nets. Factors were reef type (dead margin or reference; fixed), site nested within reef type (random), and month (fixed).

Source	df	Mean Square	F	Significance
Reef type	1	356.001	0.281	0.624
Site (Reef type)	4	1267.778	4.430	0.002
Month	11	2379.484	10.278	<0.001
Residual	343			



Chordata	<i>Momphus</i>	Stone crab	3	1.6 ± 0.5 (1.0–2.1)	2.5 ± 0.8 (1.7–3.3)	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
	<i>maculatus</i>	Grass shrimp	610			1	0	2	14	28	261	52	92	66	18	44	32																
	<i>Palaemonetes</i>	Atlantic mud crab	534	1.8 ± 0.1 (1.4–2.1)	3.3 ± 0.3 (1.5–4.8)	70	72	51	24	28	59	47	21	48	38	33	43																
	<i>Palaemonetes</i>	Pink shrimp	145	4.6 ± 0.5 (2.8–6.5)	1.3 ± 0.3 (0.3–2.4)	0	3	9	5	3	6	5	0	0	0	0	18																
	<i>Palaemonetes</i>	Green porcelain crab	584	0.7 ± 0.1 (0.6–0.8)	0.5 ± 0.1 (0.3–0.6)	383	54	29	9	15	36	16	6	14	9	4	9																
	<i>Palaemonetes</i>	Harris's mud crab	243	0.9 ± 0.1 (0.5–1.4)	0.6 ± 0.1 (0.2–1.0)	0	6	17	26	17	46	11	12	50	15	13	30																
	<i>Palaemonetes</i>	Common mantis shrimp	1	10.0	18.0	0	0	0	0	0	0	0	0	0	0	0	0																
	<i>Palaemonetes</i>	Sheepshead	21	7.2 ± 0.1	12.7 ± 1.9	3	1	1	7	0	0	0	4	0	0	0	3																
	<i>Palaemonetes</i>	Silver perch	7	4.6 ± 0.2 (4.0–5.0)	1.5 ± 0.2 (1.0–2.0)	3	0	0	3	0	0	0	0	0	0	0	1																
	<i>Palaemonetes</i>	Trillium goby	1	4.0	0.8	0	0	0	0	0	0	1	0	0	0	0	0																
	<i>Palaemonetes</i>	Florida blenny	6	4.3 ± 0.2	1.2 ± 0.4	0	0	0	1	3	1	0	0	0	0	0	0	1															
	<i>Palaemonetes</i>	Sheepshead minnow	3	5.5 ± 0.7 (4.1–6.5)	3.1 ± 1.1 (1.1–4.8)	1	0	0	0	1	1	0	0	0	0	0	0	0															
	<i>Palaemonetes</i>	Irish pompano	1	7.3	10.9	0	0	1	0	0	0	0	0	0	0	0	0	0															
	<i>Palaemonetes</i>	Gold-spotted killifish	1	6.8	7.8	0	1	0	0	0	0	0	0	0	0	0	0	0															
	<i>Palaemonetes</i>	Gulf killifish	2	8.8 ± 1.7 (7.1–10.5)	11.1 ± 6.9 (4.2–17.9)	0	0	0	0	0	1	18	19	3	0	1	2	0	7														
	<i>Palaemonetes</i>	Darter goby	54	3.5 ± 0.3 (2.2–5.0)	0.5 ± 0.2 (0.2–1.2)	0	0	1	12	49	189	27	6	3	4	12	3	8															
	<i>Palaemonetes</i>	Naked goby	736	3.1 ± 0.2 (2.6–3.8)	0.6 ± 0.1 (0.3–1.0)	13	23	24	31	165	228	62	47	54	28	32	29																
	<i>Palaemonetes</i>	Code goby	267	2.9 ± 0.2 (2.3–4.5)	0.5 ± 0.2 (0.1–1.5)	1	3	12	49	189	27	6	3	4	12	3	8																
	<i>Palaemonetes</i>	French grunt	34	3.7 ± 0.3 (2.1–5.5)	1.1 ± 0.2 (0.1–3.0)	0	0	0	0	0	0	0	0	0	0	0	0	0															
	<i>Palaemonetes</i>	Pinfish	148	3.8 ± 1.0 (2.3–7.7)	1.8 ± 1.1 (0.1–8.3)	3	1	2	0	1	0	0	0	5	1	11	0	5	4	47	11												
	<i>Palaemonetes</i>	Rainwater killifish	84	2.5 ± 0.3 (2.0–3.3)	0.3 ± 0.1 (0.1–0.5)	0	0	0	0	0	0	0	0	0	0	0	0	0															
	<i>Palaemonetes</i>	Gray snapper	25	5.9 ± 1.1 (3.0–11.2)	6.5 ± 3.0 (0.6–21.3)	1	0	1	1	10	2	4	0	2	1	3	0																
	<i>Palaemonetes</i>	Striped mullet	2	2.0 ± 1.0 (2.1–23.0)	13.4 ± 0.6 (12.8–14.0)	0	0	1	0	0	1	0	0	0	0	0	0	0															
	<i>Palaemonetes</i>	White mullet	1	11.5	16.0	0	0	0	0	0	0	0	0	0	0	0	0	0															
	<i>Palaemonetes</i>	Oyster toadfish	40	6.9 ± 1.2 (2.3–9.0)	7.8 ± 2.3 (0.2–13.3)	4	1	5	0	5	0	1	0	3	5	8	8																
	<i>Palaemonetes</i>	Gulf flounder	1	4.9	1.1	0	0	0	0	0	0	0	0	0	0	0	0	0															
	<i>Palaemonetes</i>	Southern flounder	1	3.6	0.4	0	0	0	0	0	0	0	0	0	0	0	0	0															
	<i>Palaemonetes</i>	Saffron molly	109	4.8 ± 0.4 (4.3–5.6)	2.1 ± 0.4 (1.6–3.0)	1	0	2	0	75	23	6	2	0	0	0	0	0															
	<i>Palaemonetes</i>	Gulf pipefish	16	6.6 ± 0.4 (5.6–8.6)	0.2 ± 0.1 (0.1–0.4)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	13												

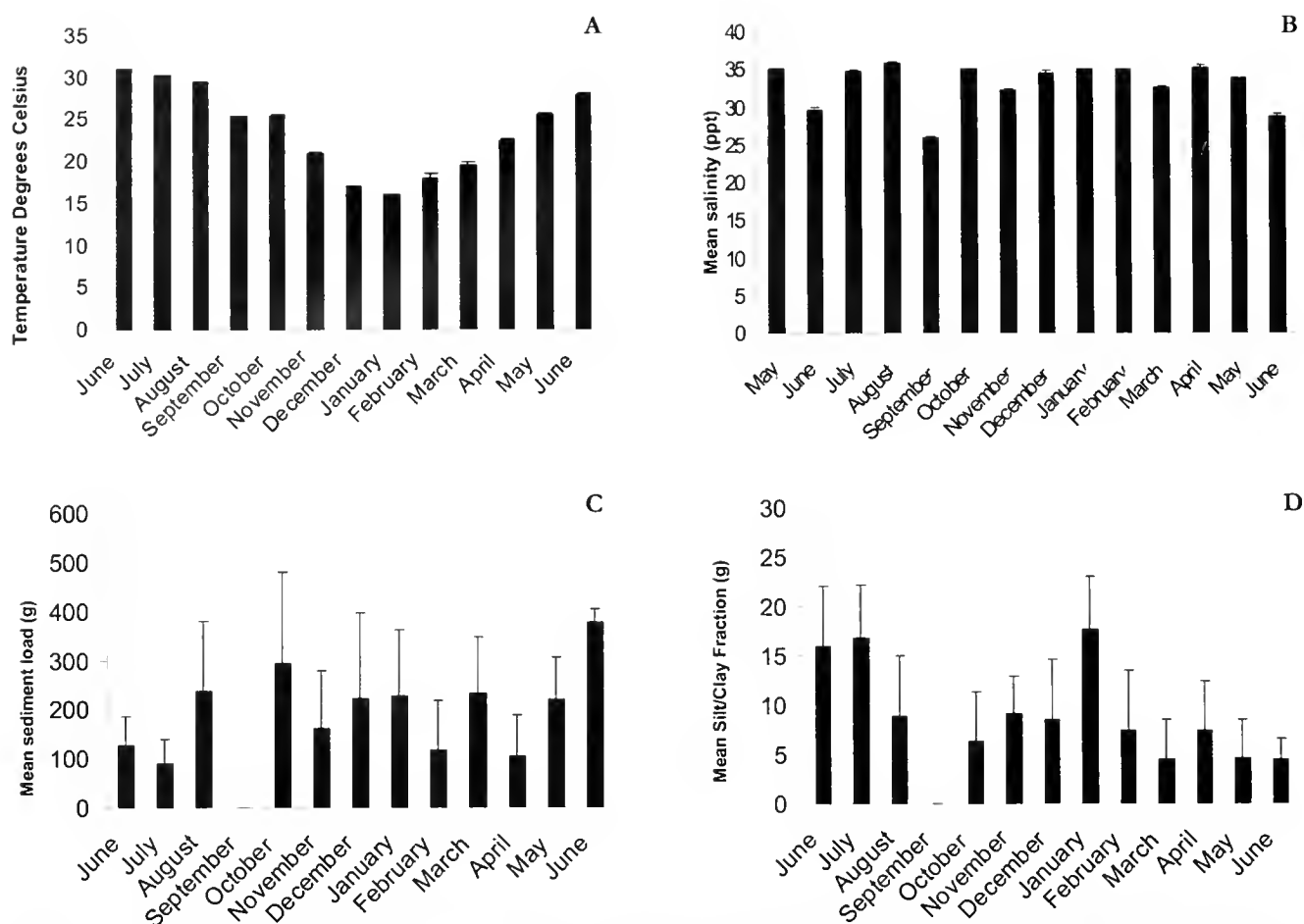


Figure 5. Abiotic variables (a). Mean monthly temperatures ( $\pm$  SE) at lift net sampling dates in Mosquito Lagoon. (b) Mean salinities ( $\pm$ SE) of Mosquito Lagoon on lift net sampling dates. (c) Mean sediment load ( $\pm$ SE) per month from June 2004 to June 2005. (d) Mean silt/clay percentage ( $\pm$ SE) per month from June 2004 to June 2005.

One individual of the invasive Asian green mussel *Perna viridis*, which has devastated some oyster reefs in Tampa Bay, Florida (Baker et al. 2003), was also recently found on a piling in Mosquito Lagoon (MB pers. obs.). It has not been found on Mosquito Lagoon oyster reefs to date. Both nonnative bivalves continue to be monitored within the IRL.

#### Motile Macrofauna

The two most abundant motile species sampled within the lift nets were the bigclaw snapping shrimp *Alpheus heterochaelis*

(2,489 individuals) and the flat mud crab *Eurypanopeus depressus* (1,217 individuals) (Table 5). Previous studies found these two species to be present in temperate waters on both the Atlantic coast of North Carolina (132 individuals of *E. depressus*; Glancy et al. 2003) and the gulf coast of Florida (3,184 individuals of *E. depressus*, 364 individuals of *A. heterochaelis*; Meyer 1994). Similar to Tolley et al. (2005), we found the replacement of temperate species by tropical congeners, including the replacement of the striped blenny *Chasmodes bosquianus* in the Northern Atlantic (Breitburg 1999, Coen et al. 1999) by the Florida blenny *Chasmodes saburrae*.

TABLE 8.

Three-factor nested ANOVA comparing total sediment loads collected per month at lift net sites. Factors were reef type (dead margin or reference; fixed), site nested within reef type (random), and month (fixed).

Source	df	Mean Square	F	Significance
Reef type	1	317305.223	1.961	0.234
Site (Reef type)	4	161861.650	3.357	0.011
Month	11	124938.063	2.624	0.004
Residual	199			

TABLE 9.

Three-factor nested ANOVA comparing silt/clay fractions collected per month at lift net sites. Factors were reef type (dead margin or reference; fixed), site nested within reef type (random), and month (fixed).

Source	df	Mean Square	F	Significance
Reef type	1	39.068	0.687	0.454
Site (Reef type)	4	56.862	0.871	0.482
Month	11	409.454	9.004	<0.001
Residual	199			



The fifth most abundant mobile species was the green porcelain crab, *Petrolisthes armatus* (Table 5). It is considered an invasive exotic along the South Atlantic Bight (Knott et al. 1999, Glaney et al. 2003). Populations of this species can historically be found in the Pacific (i.e., California to Peru) and the Atlantic (i.e., Africa, Ascension Island, Bermuda, Bahamas, Gulf of Mexico, West Indies, Caribbean and South America down to Brazil; Knott et al. 1999). Although the pathway of introduction remains unknown, ballast transport and increasing winter temperatures, which favor its establishment are possibilities (Knott et al. 1999). It was first collected along Florida's east coast in the 1930s in Biscayne Bay and Miami Beach (Knott et al. 1999). Slowly, it spread northward, becoming well established in the Indian River Lagoon system (Knott et al. 1999). Studies have shown abundances to increase dramatically in only a few years after introduction (Knott et al. 1999). The current range of *P. armatus* along the South Atlantic Bight stretches from South Carolina down to the southern tip of Florida (Knott et al. 1999).

Size and biomass data for several crustacean and fish species revealed that both juvenile and adult individuals were present on the reefs (Table 5). For example, large ranges were seen for the big-claw snapping shrimp *Alpheus heterochaelis* (length 1.6–2.5 cm; biomass 0.3–0.5 g), blue crab *Callinectes sapidus* (length 2.3–4.3 cm; biomass 1.5–6.6 g), stone crab *Menippe mercenaria* (length 1.0–2.1 cm; biomass 1.7–3.3 g), grass shrimp *Palaemonetes vulgaris* (length 2.5–3.8 cm, biomass 0.3–0.6 g) and pink shrimp *Penaeus duorarum* (length 2.8–6.5 cm, biomass 0.3–2.4 g) (Table 5). Within the Chordata family, different life stages were seen for the sheepshead *Archosargus probatocephalus* (length 4.4–9.7 cm, biomass 8.5–19.8 g), pinfish *Lagodon rhomboides* (length 2.3–7.7 cm, biomass 0.1–8.3), gray snapper *Lutjanus griseus* (length 3.0–11.2 cm, biomass 0.6–21.3 g), oyster toadfish *Opsanus tau* (length 2.3–9.0 cm; biomass 0.2–13.3 g), and numerous killifish and goby species (Table 5).

The blue crab *Callinectes sapidus*, the pink shrimp *Penaeus duorarum* and juvenile forms of several important finfish species were collected in the lift nets within Mosquito Lagoon (Table 5). Hence, commercially and recreationally valuable species are utilizing oyster reefs within Mosquito Lagoon, confirming the importance of oyster reefs to the economy of this region. These species were also found to be utilizing oyster reefs on the west coast of Florida (Tolley et al. 2005).

Additional comparisons can be made with the motile species found on oyster reefs in Mosquito Lagoon to lift net studies of intertidal reefs on the west coast of Florida (26° 25'56"N, 81° 48'34"W) (Tolley et al. 2005, Tolley & Volety 2005). Salinities and temperatures found in Mosquito Lagoon (mean: 33 ppt, 23.8°C) are comparable to the system studied in southwest Florida (mean: 32.5 ppt, 27.1°C). Overall species richness (the total number of species found per net) was found to be similar between the two different Florida locations (Gulf: 4–11 species/month versus Mosquito Lagoon: 4–11 species/month), whereas density was slightly lower in Mosquito Lagoon (Gulf: 20–400 organisms/net versus Mosquito Lagoon: 12–40 organisms/net). In both systems, there were more fishes than decapod crustacean species (Gulf: 16 versus 9, Mosquito Lagoon: 23 versus 18). In both locations, decapod crustaceans dominated all motile samples numerically.

#### Dead Margins Affect on Oyster Reef Communities

Dead margins, attributed to wakes from recreational boating in Mosquito Lagoon (Grizzle et al. 2002, Wall et al. 2005), did not

have a significant effect on the back-reef usage of oysters as substrate by either sessile or motile species (Tables 3, 4, 6 and 7). The back-reef areas of both were also visually very similar. This suggests the back-reef areas on oyster reefs with dead margins function similarly to a reference oyster reef with no dead margin.

Although dead margins did not have an impact on richness or density of organisms found, sessile organisms preferred to settle on living oyster clumps rather than on the disarticulated shells placed within the nets (Fig. 4). The 3-dimensional structure of the two settlement substrates was very different. Disarticulated shells were single and loose, laid flat on the benthos, and were often covered by sediment. These shells were frequently displaced by water motion. Live oysters attached together to form clusters and rarely moved. These 3-dimensional clusters probably provided more protection and refuge from predators for sessile inhabitants than the 2-dimensional, disarticulated shells. Subsequent research has shown that reference reefs contained twice more oyster clumps than reefs with dead margins within Mosquito Lagoon (Stiner 2006). Combined, these results reveal a new negative impact of dead margins on sustaining biodiversity in Mosquito Lagoon.

Wall et al. (2005) found an increase in sediment accumulation on the seaward edges (fore-reef areas) of reefs with dead margins in Mosquito Lagoon and suggested this was caused by sediment resuspension associated with large numbers of boat wakes. Increased sediment has been shown to decrease the settlement of *Crassostrea virginica* (Boudreaux 2005). Thus, any difference in sediment loads between locations would have been predicted to have an effect on species assemblages between the two types of reefs (reference and dead margins). However, this study focused exclusively on the back-reef regions of oyster reefs and did not show any differences in sediment loads between reef types. Dead margins are hypothesized to protect these back-reef areas by preventing sediment accumulation.

During this study we documented the usage of intertidal oyster reefs in Mosquito Lagoon by 105 different species. This included 76 invertebrates and 29 chordates. The richness in diversity found within the reefs of *Crassostrea virginica* is comparable with other systems in the Indian River Lagoon system. A study of decapods associated with seagrass communities in the Indian River Lagoon showed remarkable diversity. In all, 38 decapod species were found in seagrass beds (Gore et al. 1981; Smithsonian Institution 2006) as compared with the 19 decapod species we found using oyster reefs (Tables 2 and 5). These examples demonstrate the extremely high diversity in the IRL that can be attributed to its important habitats, including seagrass beds and oyster reefs. The data from this study are an important step to gaining a better understanding of these oyster reefs and their essential role in the estuary. Additionally, this data provides a baseline from which to evaluate efforts to practice sustainable ecosystem management of *Crassostrea virginica* within Mosquito Lagoon.

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## A DECISION SUPPORT TOOL FOR SHELLFISH MANAGEMENT IN MISSISSIPPI SOUND

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**ABSTRACT:** The Eastern oyster (*Crassostrea virginica*) is an economically important fisheries resource along the US Gulf Coast and the eastern seaboard. In the past few years, Mississippi has ranked second in the country in shellfish production, harvesting an average of 350,000 sacks (~15.8 million kg of oysters) per year. A GIS-based decision support tool was developed to aid the Mississippi Department of Marine Resources in managing shellfish in the state. The tool was designed using the ESRI ArcGIS 8.3 Arcview software, which enabled us to integrate GIS data layers (e.g., oyster reef sites) with a program that automatically obtains Pearl River stage (gage height) and rainfall amount data from the USGS and NOAA websites, respectively. The program then compares the data with established area-specific standards and makes recommendations to the shellfish manager on the area(s) that should be closed or opened. The decision support tool is the first tool developed for shellfish management in the US Gulf Coast area. Although it was developed for use in Mississippi, it can be modified for use in other states. It simplifies the shellfish management process and maintains a database of the water quality data and the management actions that have been taken, thereby facilitating data analyses and preparation of reports.

**KEY WORDS:** oyster management, Mississippi Sound, river stage, precipitation, GIS data layers

### INTRODUCTION

The Eastern oyster (*Crassostrea virginica*, Gmelin 1791) is one of the economically important fisheries resources in the eastern seaboard of the United States and the US Gulf Coast region, and it accounts for more than 60% of all oysters harvested in the United States (Gore 1992). In the past few years, Mississippi has ranked second in the country in shellfish production, and harvested an average of 350,000 sacks (~15.8 million kg of oysters) per year.

*Crassostrea virginica* inhabits shallow waters in intertidal and subtidal zones, and their survival and production depend chiefly on phytoplankton productivity, flooding, salinity and temperature variations, levels of parasitism by *Perkinsus marinus* (dermo) and *Haplosporidium nelsoni* (MSX) and predators (Andrews et al. 1959, Menzel et al. 1957, Soniat et al. 1989, Powell et al. 1995, Jordan 1995). Although *C. virginica* tolerates a wide range of salinity with lower and upper limits of about 2 ppt and 39 ppt, respectively (Shumway 1996), high salinities and warm temperatures are conditions that favor the major predators and parasites of the shellfish. For example, the conch, *Strombula haemastoma* thrives at salinities >12 ppt and can have significant predatory impacts on the oyster population (Butler 1954, Gagliano et al. 1970). Another major cause of mortality of oysters in high salinity areas and during warm months along the Gulf of Mexico is *P. marinus*, a parasitic protozoan (Ogle & Flurry 1980, Ray 1996, Soniat 1996). The impacts of *P. marinus* parasite on oysters depend on local as well as regional variations in climatic factors (Powell et al. 1992, Kim & Powell 1998).

Management of shellfish is aimed at enhancing the habitat via cultch planting and cultivation, harvest control, and minimizing the risks to humans of consuming raw contaminated shellfish. Disease outbreaks resulting from the consumption of oysters contaminated by bacterial (e.g., *Vibrio vulnificus* and *Vibrio parahaemolyticus*) and viral pathogens (Leonard 2001, see also a review by Rose et al. 2001) have been reported in other states. The levels of these pathogens depend on climatic factors such as water temperature, intensity of solar radiation and precipitation. The latter is usually accompanied by runoff carrying pollutants into coastal waters (Lipp et al. 2001). To reduce the risks of acquiring diseases caused by consumption of contaminated oysters, the United States Food and Drug Administration and the Interstate Shellfish Sanitation Conference (ISSC) formed the National Shellfish Sanitation Program (NSSP) that developed criteria for the protection of shellfish harvesting waters (ISSC et al. 1999). For example, the Mississippi Ordinance 1.016 section 15.4.1.3, requires a shellfish harvesting area to be closed "when the geometric mean of the seawater from compliant sampling stations in the area exceed a fecal coliform most probable number (MPN) of 14 per 100 mL and/or more than 10% of the samples exceed a MPN of 43 for a 5-tube 3 dilution test."

In Mississippi, rainfall amounts and/or Pearl River stage are used as metrics for closing shellfish harvesting areas in Mississippi Sound. Many years of studies indicate that both factors are highly correlated with fecal coliform levels (Chigbu et al. 2004a). Each of the nine conditionally approved shellfish harvesting areas in Mississippi has specific closure criteria. These are monitored by designated MDMR personnel using sources such as telephone, various official reports, internet, as well as by direct observation, as stated in the Ordinance 1.016. The objective of our study is to design a GIS-based decision support tool that will ease the process of shellfish management in the state. Although GIS-based tools and meth-

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ods have been developed for various projects including, microbial risk assessments (Kistemann et al. 2001a, 2001b), environmental impact assessments (Gustafson et al. 2001, Sahin & Kurum 2002), flood area and damage estimation (Renyi & Nan 2002) and resource management (Webb & Bacon 1999), to our knowledge none is available for use in shellfish management in the US Gulf Coast region.

### Study Area

Mississippi Sound has a mean depth at midtide level of 3 m, and is partially separated from the northern Gulf of Mexico by a number of barrier Islands. Within the estuary, water from the Gulf mixes with water that flows in from a number of bayous and rivers. The Pearl and Pascagoula Rivers account for more than 90% of freshwater discharge into the Sound (Eleuterius 1978). The Mobile and Mississippi Rivers sometimes contribute significant freshwater into the eastern and western portions of the Sound, respectively (Orlando et al. 1993). The eastern and western parts of the Sound have more variable and lower salinities than the central part of the Sound. Under most conditions, Mississippi Sound is partially or well mixed because of tidal action and winds (Eleuterius 1978).

Pearl River is about 789 km long and drains an area of approximately 23,000 km<sup>2</sup> before emptying into the western part of the Mississippi Sound, an area that contains some of the most productive oyster populations in the Southeastern United States (Gore 1992). Studies conducted from June 1973 to February 1975 (Eleuterius 1977) suggested that the area experiences bottom minimum and maximum salinity of 2.0–4.0 ppt and 18.0–22.0 ppt, respectively.

### Oyster Management in Mississippi Sound

The Department of Marine Resources manages about 17 natural oyster reefs in Mississippi. About 97% of the harvested oysters in Mississippi come from the reefs in the western part of the Mississippi Sound. Management of the Sound for shellfish harvesting is currently based on Pearl River stage, local rainfall amounts and fecal coliform counts (Table 1). River Stage information is collected directly from the USGS website for specific river gauges. The National Weather Service web-site is monitored for rainfall from three NOAA rain gauge locations: Bay St. Louis, Waveland and Pascagoula, MS.

Historical data on fecal coliform levels during the oyster-harvesting season (typically October to April), have been used by shellfish managers to classify Mississippi Sound into areas desig-

nated as Approved, Conditionally Approved, Prohibited, Restricted or Unclassified. Harvesting of oysters for direct consumption is permitted only in areas designated as approved or conditionally approved. At present, nine areas are designated as conditionally approved, each of which has specific opening and closing criteria (Table 1), based on rainfall amounts and/or river stage as outlined in the Mississippi Ordinance 1.016. Beside heavy precipitation, other factors listed in the Ordinance that may result in temporary closures of an approved or conditionally approved area include "a hurricane, flooding, chemical spill, hazardous waste or raw sewage discharge, sinking or grounding of vessels carrying hazardous cargoes, evidence of the existence of marine biotoxins."

## METHODS

The GIS data layers that were used in the tool included the oyster reefs, shellfish management areas, sampling stations for water quality monitoring, Gulf Coast imagery, and the USGS river gauge locations. The tool was designed using the Environmental Systems Research Institute (ESRI) ArcGIS 8.3 Arcview and Microsoft Access. The ESRI package provides the user the capability to use geographic data, as well as a programming environment that integrates Visual Basic for application and the ArcObjects library. Microsoft acts as an external relational database that is used to store the metrics for each of the oyster zones as well as the current and historical gauge measurement and information data. The tool automatically obtains river gauge information from the USGS website: <http://waterdata.gov>, and the National Weather Service (NWS) precipitation data from the NOAA website: <http://www.srh.noaa.gov>. Because the gauge data are ingested from the various websites, they are parsed and stored in the corresponding table in the backend database. On a predetermined time interval, the application is designed to use the current gauge data to decide whether an area should be closed, and then uses data on fecal coliform counts to determine whether the area should be reopened. The fecal coliform information is stored in a separate Access database. The readings are stored in a database at a remote laboratory and then transferred to the cataloging database via an automated E-mail application. The tool was built to run on an IBM compatible with Windows 2000 operating system and the following specifications:

- (a) CPU speed of 450 MHz (minimum); 800 MHz or higher is recommended
- (b) A pentium or higher processor
- (c) Memory/RAM 128 MB (minimum); 256 MB or higher recommended
- (d) Swap space 300 MB (minimum)
- (e) Disk space of 695 MB FAT32 for the ESRI software, and 300 MB for data storage

## RESULTS AND DISCUSSION

The shellfish decision support tool consists of 5 major components, namely, the Shellfish Management, Manual Close, External Conditions, Rules Change and Strategic Management, the functions of which are summarized in Table 2. The display user interface for the tool (Fig. 1) is divided into 5 areas.

Area 1 contains the typical user interfaces found in normal office applications such as MSWord, MSeExcel, including "file

TABLE 1.

Criteria for closing conditionally approved shellfish harvesting areas

Area	Closing criteria
Area IB-CA	10 ft Pearl River Stage and/or 2 inches rainfall
Area II A-CA	10 ft Pearl River Stage and/or 1 inch rainfall
Area II B-CA	10 ft Pearl River Stage and/or 1 inch rainfall
Area II C-CA	12.5 ft Pearl River Stage and/or 2 inches rainfall
Area II D-CA	10 ft Pearl River Stage and/or 1 inch rainfall
Area III-CA	2 inches rainfall
Area IV-CA	10 ft Pearl River Stage and/or 1 inch rainfall
Area V-CA	2 inches rainfall
Area VIII-CA	0.75 inches rainfall

**TABLE 2.**  
Main components of the shellfish management tool and their functions

Main components	Functions
1. Shellfish management	<p>*Monitors rainfall and river gauge data and compares it to monthly rule criteria for each area.</p> <p>*Recommends closure of an area when certain criteria have reached the threshold defined in the Shellfish Management Plan.</p> <p>*Allows for fecal coliform counts to be analyzed, and determines when it is safe to re-open an area for oystering.</p>
2. Manual close	<p>*Provides DMR officials with the ability to allow some other closing conditions such as oil spills and hurricanes which are not automatically monitored by the application.</p> <p>*When an area has been manually closed, it can only be manually reopened.</p> <p>*The manual close function prohibits or "locks-out" normal operations of the shellfish management function for the area(s) normally closed.</p>
3. External conditions	<p>*Allows DMR officials to close for other specified reasons, hence it is similar to the Manual close function.</p> <p>*Allows latitude and longitude coordinates to be associated with the closing event.</p>
4. Rules change	<p>*Allows the tool to remain up-to-date with amendments to Ordinance 1.016 by making it possible for DMR officials to change any closing or opening criteria (e.g. river stage levels, rainfall amounts, geometric mean MPN level) for any of the conditionally approved areas.</p>
5. Strategic management	<p>*Used for longer term (e.g., 1 year) evaluation of the shellfish harvesting areas based on fecal coliform counts, in order to determine whether a re-classification of oyster-harvesting areas is necessary.</p>

save, exit, copy and paste," in addition to some ArcInfo 8.2 functionality.

Area 2 contains the graphical user interfaces (GUI) for the Shellfish Management Application. The tool bar has buttons, each of which operates a separate function within the application.

- (a) The Shellfish Management function button continually monitors river stage and precipitation and automatically recommends whether an oyster harvesting area should be closed. The program's recommendations are reviewed by the DMR officials who then decide whether to accept or reject the recommendations.

When the data being monitored by the application indicate a status change to a Shellfish Management area, a Shellfish Management Form (Fig. 2) automatically opens. The form has 5 areas that show Program Recommendations, National Weather Service (NWS) data comprising information on rain gauges at Waveland, Bay Saint Louis

and Pascagoula, MS and the US Geological Survey (USGS) Pearl River stage data at Pearl River, Louisiana. In addition, the form has a time stamp that shows the latest time that the external data from the gauges were collected from external websites by the shellfish application. Finally, the form has an error message display area. If no value is found for rainfall, an error message will notify the user in the area, and if the value is invalid, it will be presented as "-9999" in the table.

When the program recommendations button is selected from the Shellfish Management Form, the program recommendations form opens (Fig. 3) to allow DMR staff to review the reasons why the application is recommending a status change. The application's recommendation can then be accepted or rejected by the DMR staff.

There are 5 areas on the Program Recommendations Form: (1) Area Tabs; (2) Recommendation; (3) Reason for Recommendation; (4) Reason for Rejection and (5) Process Recommendation.

The area tabs consist of nine separate and distinct tabs; each tab contains information specific for a particular area. If the tab heading is in bold, it indicates the particular area has a recommended status change otherwise, no status change recommendation exists.

The recommendation tab has a title that shows either Recommend Open or Recommend Close, which is associated with check boxes for use by DMR staff to indicate an acceptance or rejection of the application's recommendation. The reason for recommendation tab describes why the application is recommending a change for the area. The reason for rejection tab is for use by DMR officials to record their reasons for rejecting a recommendation made by the tool. The reason will be stored in the database and can be reviewed by the current status or history reports functions. The process recommendation tab is selected to update the display with the current status (whether accepted or rejected). If a status change was recommended and this button is not selected, the display will not reflect the status changes. This tab should be selected after all areas have been reviewed and recommendations are either accepted or rejected. The map display will reflect the changes accepted (e.g., Fig. 4). The program "recycles" every four hours when more information is received from the USGS and NWS websites. If an area is not closed, even though the application recommends it, the area will again be recommended to close when the program recycles in four hours.

- (b) The external conditions buttons are used to close and re-open areas for events that are not routinely monitored by the Shellfish Management application. The add external conditions allows an event to be pinpointed on the map with either latitude/longitude coordinates or a mouse-click on the screen. The delete external conditions button removes external conditions, which have been previously established.

When the external conditions buttons are selected, the external conditions form (Fig. 5) opens. This form consists of nine areas: (1) Event Location; (2) Area Name and Present Status; (3) Date/Time; (4) Latitude/Longitude; (5) New Status; (6) Open/Close All Zones; (7) External Condition; (8) Operator Comments and (9) Set Status.

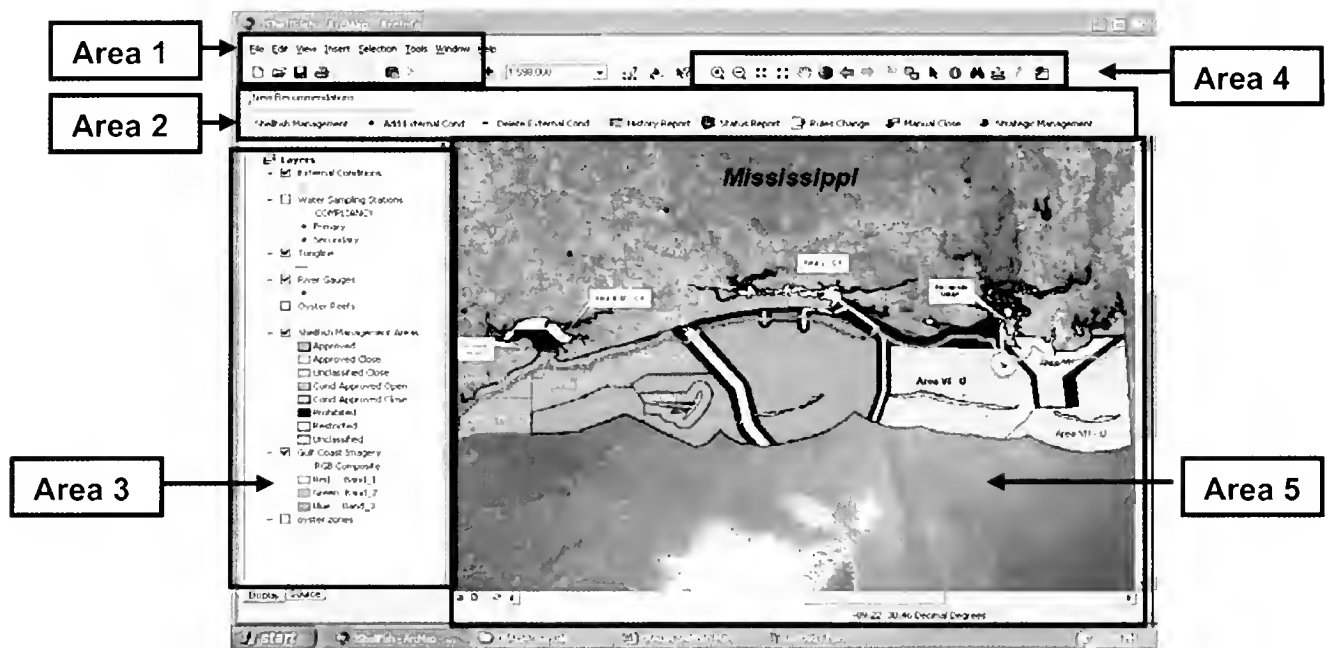


Figure 1. The display user interface for the Shellfish Management Tool

The event location button allows one to identify the location of an event by selecting entered mouse click or entered by coordinate value. The area name and present status button lists all of the areas and their present statuses. The area(s) can be selected by left-clicking the mouse. To select more than one area, hold down the shift key while

selecting the area with the mouse. Areas that are selected are highlighted.

The date and time stamp is used to record the time and date that the manual close function is used. This is recorded for the current status and history reports.

The longitude and latitude buttons are used to display

## Program Recommendations

## USGS River Gauge & NWS Rainfall Data

## Time Stamp

Shellfish Management

VERMILION  
HONN  
Shellfish Management System

Program Recommendation(s)

Involved Bay Saint Louis Rainfall Value

Waveland/Bay Saint Rain Fall Gauge	Pascagoula Rain Fall Gauge	Pearl River River Gauge	National Weather Service
Current Value 2.81 4/1/2003 2:00:00 PM	Current Value 0.64 4/1/2003 3:14:00 PM	Current Value 19.98 5/5/2003 6:30:00 AM	Bay St. Louis -9999
Period High 2.81 4/1/2003 2:00:00 PM	Period High 0.79 4/1/2003 11:29:00 AM	Period High 19.98 5/5/2003 6:30:00 AM	Pascagoula 0.25
Period Low 1.85 4/1/2003 7:00:00 AM	Period Low -0.01 3/31/2003 3:44:00 AM	Period Low 9.91 5/5/2003 2:30:00 AM	Waveland 0.25

Pearl River Data Current as of 5/5/2003 6:30:00 AM  
Pascagoula Rainfall Data Current as of 4/1/2003 3:14:00 PM  
Bay St. Louis / Waveland Rainfall Data Current as of 4/1/2003 2:00:00 PM  
National Weather Service Data Current as of 4/10/2003 11:16:00 AM

## Error Message Display

Figure 2. The Shellfish Management Form



The diagram illustrates the Shellfish Program Recommendations Form. It features a tabbed interface with tabs labeled Area I B, Area II A, Area II B, Area II C, Area II D, and Area IV. The form is divided into several sections:

- Recommendation:** A section with radio buttons for "Recommend Close", "Accept Recommendation", and "Reject Recommendation".
- Reason for Recommendation:** A text area for providing the reason for a recommendation.
- Reason for Rejection:** A text area for providing the reason for rejecting a recommendation.
- Process Recommendation:** A button at the bottom of the form.

Callout boxes point to these specific sections: "Recommendation" points to the radio buttons, "Reason for Recommendation" points to the top text area, "Reason for Rejection" points to the bottom text area, and "Process Recommendation" points to the bottom button.

Figure 3. The Shellfish Program Recommendations Form

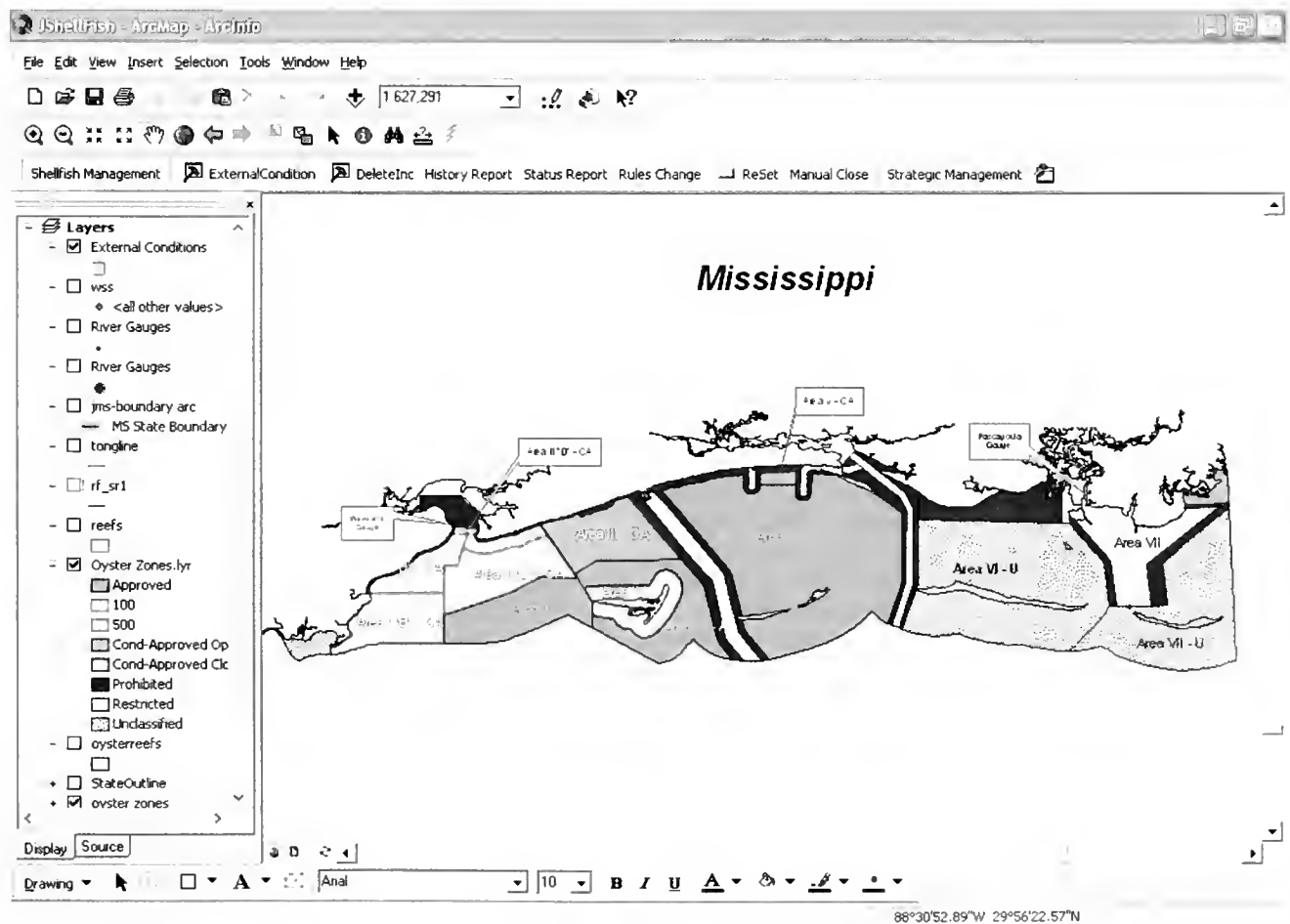


Figure 4. The Shellfish Program Map Display area showing the closed conditionally approved shellfish harvesting areas highlighted in red color.

**Event Location**

**Area Name & Present Status**

**Date & Time Stamp**

**Longitude & Latitude Values**

**New Status**

Area Names	Present Status
Area I "A" - U	unclassified
Area I "B" - CA	conditionally appr
Area II - A	approved
Area II "A" - CA	conditionally appr
Area II "B" - CA	conditionally appr
Area II "C" - CA	conditionally appr
Area II "D" - CA	conditionally appr
Area III - A	approved
Area IV - CA	conditionally appr
Area V - A	approved

**Open/Close All Zones**

**External Condition**

**Operator Comments**

**Set Status**

Figure 5. The Shellfish Management External Conditions Form.

the longitude and latitude of where the external conditions symbol is on the map display.

The new status button allows the selection of an open or close status of the areas selected in the area names window. Selection is made by clicking on the arrow, which prompts a drop-down menu with Open and Closed to open.

The Open/Close all zones button allows one to select and close or open all areas in the area names list.

The External Condition button allows for the selection of the type of external condition that has warranted closure of an area.

The Operator Comments area is provided to allow additional comments to be noted. These comments are retained for the report features.

The Set Status button is clicked after all the external conditions have been established to process the decision.

- (c) The history report button allows DMR to generate reports of the opening and closing events that have taken place for a particular area, including the reasons for change in status.
- (d) The status report button generates a visual and/or a printed

report for the current status of each of the shellfish harvesting areas.

- (e) The rules change button allows the monthly rule tables to be changed for any of the shellfish harvesting areas. Rain-fall amount, river gauge levels, fecal coliform counts, and the values that determine river crest and river rise can be changed using this function.

When the rules change button is selected, the rules change form (Fig. 6) will open and is used to change the criteria for each shellfish management area to open or close, when approved by the authorized person at DMR. There are 6 areas on the form: (1) Select Area ID; (2) Area Information (for area ID); (3) River Gauge Information; (4) Rain Gauge Information; (5) Area Information (for river crest and rise) and (6) Form Navigation. The rules change function is password protected.

The select area ID allows one to select the area that requires a change in its rule criteria. The area information shows the area ID and the seasonal condition rule (area classification status).

**Select Area ID**

**Area Information**

**River Gauge Information**

**Rain Gauge Information**

**Area Information**

**Navigation**

**Oyster Reef Closure Rules Change**

**Select Area ID**

**Area Information**

**Area ID** Area II "C" - CA

**Conditions** conditionally approved

**River Gauge Information**

**ID** 02492600 **Name** Pearl River, LA

**Agency** USGS **Units** Feet **Limit** 12.5 **Period** 0

**Rain Gauge Information**

**ID** 301932089193120 **Name** Waveland, Bay Saint

**Agency** NWS **Units** Inches **Limit** 2 **Period** 24

**Area Information**

**Crest Value Change** 0.04 **ReRise Value** 0.15

**Acceptable Geometric Mean** 14

**Previous** **Next** *Record 4 of 9*

Figure 6. The Shellfish Management Rules Change Form.

The river gauge information gives information about the river gauge, such as the agency that it belongs to, as well as the ID, name, and the measurement units. It also has the limit box used to set the maximum height of the river allowed before closing a shellfish area, and the period box

used to set the maximum time (in hours). In Figure 6, for example, Area IIC-CA is recommended to close when the Pearl River gauge reaches 12.5 ft.

The rain gauge information gives details about the rain gauge, such as agency, ID and name and measurement

units. The limit box within the rain gauge information area is used to set the maximum amount of rain (in inches) allowed before closing a shellfish area. The period box is used to set the maximum time (in hours) of rain accumulation. For example, in Figure 6, Area IIC-CA is recommended to close when the Waveland rain gauge shows that it has rained 2 inches in the last 24 h period.

The area information section displays the values the application uses for river crest and *re-rise*. In addition, the maximum acceptable geometric mean fecal coliform count is set here. The crest value change is used to determine when the application considers a river crest has occurred. It considers the river to have crested if the level has dropped 0.04 ft below the highest river value recorded since closure. The *ReRise* value is used to set the application's river's *re-rise* condition. The river is considered to have reached a level of "*re-rise*" (which could indicate a condition to close the area again) if the river stage is 0.15 ft above the level that caused the closure. The acceptable geometric mean area is used to set the maximum value for fecal coliform count allowed to keep a shellfish management area open for harvesting.

The navigation area allows the operator to navigate through the rules change records by clicking on the previous and next buttons on the bottom of the form.

- (f) The manual close button, like the external condition function, allows the program to be operated outside of the scope of the shellfish management rules closure recommendations. The rules closure criteria affect only the conditionally approved areas. In contrast, the manual close function allows any area to be closed.
- (g) The strategic management button allows for a year-end evaluation of the shellfish harvesting areas, based on calculations of the geometric means of fecal coliform counts. Fecal coliform data of water samples from each area are used by the application to calculate the geometric mean of the fecal coliforms for each water sample station. These results can then be used by DMR to reclassify areas as approved or conditionally approved. An area can be classified as approved "if the bacteriological quality of the water of every sampling station does not exceed a fecal coliform median or geometric mean MPN of 14 per 100 mL, and not more than 10% of the samples exceed an MPN of 43 for a 5-tube 3 dilution test" as presented in the ordinance. An area may be classified as conditionally approved if the area meets approved area standards under certain conditions specified in the areas management plan (e.g., rainfall or river stage at a particular location is less than the maximum allowed for in the management plan).

Area 3 is known as the table of contents, and shows what layers are active in the map display area.

Area 4 consists of tools used to move around the map display area.

Area 5 is the map display area, where the shellfish management areas are displayed. They will be color-coded as to their status.

Fecal coliform reports are used mainly for two reasons with regard to the shellfish management areas. First, recent fecal coliform counts are used in determining whether the shellfish harvesting areas can be reopened after a rise in river stage and/or a rain event. Second, the strategic management function of the application will evaluate all of the fecal coliform counts for an entire

season. The counts are then computed and used to re-evaluate if the shellfish management area should be classified as approved or conditionally approved. DMR collects water samples from various stations, which are then analyzed for fecal coliforms at the US FDA approved Gulf Coast Research Laboratory, Ocean Springs, Mississippi. The fecal coliform data are sent to DMR via e-mail. The shellfish management application was designed to perform a semiautomatic ingestion of the newly delivered data into the DMR database and also calculate the geometric mean fecal coliform counts to recommend whether to reopen the reefs.

The Pearl River has much influence on the water quality in the western part of the Mississippi Sound, hence Pearl River stage along with rainfall amount are two important variables that the shellfish management tool monitors for closing shellfish harvesting areas. Pearl River stage varies seasonally (Chigbu et al. 2005a), with a peak in late winter/early spring and a minimum in summer/early fall. The river stage also varies between years because of the influence of ENSO events on precipitation (Chigbu et al. 2004). Both seasonal and interannual variations in Pearl River stage are accompanied by increased levels of fecal coliforms, which in turn determine the duration that the conditionally approved shellfish harvesting areas are opened for shellfish harvesting. In fact, Pearl River stage explains as much as 90% of the variations in fecal coliform concentrations in the western part of the Sound (Chigbu et al. 2004).

Fecal coliform levels often peak in water after rain events. In Mississippi Sound, fecal coliform levels peak within 48 h of a major local rain event (>0.5 inches), whereas Pearl River stage typically crests within about 96 h of a rain event (Chigbu et al. 2005b). It is therefore, reasonable to use the total amount of rainfall within a period of 24 consecutive hours for determining whether an area should be closed in the shellfish management tool.

To our knowledge this is the first decision support tool developed for shellfish management in the US Gulf coast area. Although it was developed for use in Mississippi, it can be easily modified for use in other states. It is easy to use; it simplifies the process of closing and opening of the conditionally approved shellfish harvesting areas, and it maintains a time series database of the water quality on which closing and opening of reefs are based as well as management actions that have been taken. It thus facilitates data analysis and preparation of reports. The tool was installed and successfully tested at DMR during the 2003/2004 shellfish harvesting season. Plans are underway to adapt the tool for use in other states.

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## CONSUMER EVALUATION OF DIPLOID AND TRIPLOID PACIFIC OYSTERS SUBJECTED TO HIGH PRESSURE TREATMENT

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**ABSTRACT** A sample of Australian oyster consumers evaluated the sensory quality of diploid and triploid Pacific oysters subjected to high pressure (HP) treatment with and without salt. Triploid and diploid oysters received comparable hedonic ratings despite having dissimilar condition indices at the time of testing. The HP treatment did not adversely affect the sensory quality of Pacific oysters because the test products received similar ratings of sensory quality to the control (unprocessed) products. Moreover, oysters subject to HP received higher ratings for liking of appearance and were judged to have lower levels of perceived saltiness compared with the control. The use of HP in extending the shelf life of farmed Australian oysters should be investigated.

**KEYWORDS:** Pacific oyster, high pressure, triploid, consumer sensory evaluation, sensory quality, *Crassostrea gigas*

### INTRODUCTION

Commercial production of triploid Pacific oysters *Crassostrea gigas* on the West Coast of North America began in 1985 and now accounts for over 30% of all Pacific oysters farmed there (Nell 2002). The very low fecundity of both chemically induced and natural triploids (Guo & Allen 1994, Gong et al. 2004) make them attractive in areas such as Port Stephens, New South Wales (NSW), Australia, where excessive overcatch makes oyster farming difficult (Nell, 1993). Therefore, because of their potential to reduce overcatch and increase growth rates, the benefits of farming triploid Pacific oysters in Port Stephens was evaluated from February 2002 to February 2004. Live Pacific oysters have a typical shelf life of 7–10 days, when refrigerated at around 4°C, whereas live Sydney rock oysters *Saccostrea glomerata*, have a shelf life of 2–3 wk, when kept cool at 8°C to 15°C (Nell, 2001). Therefore, increasing refrigerated shelf-life of shucked Pacific oysters (Calik et al. 2002), would assist the marketability of this product.

High Pressure (HP) treatment is capable of inactivating spoilage microorganisms and as a result it is currently being used as an alternative to heat treatment for food preservation. Unlike heat treatment, HP has potential to preserve the original nutrient and sensory quality of liquid and semisolid foods by maintaining covalent bond structure during a treatment that is instantaneous and uniform (Farr 1990, Kelly 2000). Therefore, HP presents a number of potential benefits for the preservation of foods and offers a number of benefits when prolonging the shelf-life of ready-to-eat refrigerated foods (Stewart & Cole 2001). Previous studies in the US have found HP treatment to be a useful tool for shucking oysters, as well as providing an extension in the refrigerated shelf-life of the product (Calik et al. 2002, He et al. 2002). HP has also improved the appearance of oysters (López-Caballero et al. 2000) and treated oysters were believed to be slightly "juicier" (López-Caballero et al. 2000), perhaps because of the increased moisture content of HP treated oysters (Cruz-Romero et al. 2003, 2006). The flavor of HP treated oysters may be enhanced, because of the uptake of salt from the water in which oysters were treated (Hoover et al. 1989).

This study was conducted to determine the influence of HP treatment, with or without the addition of salt, on consumer ap-

preciation (appearance, odor, texture, flavor) of diploid and triploid Pacific oysters. In doing so, the sensory quality of the HP treatments was compared with the quality of the untreated control.

### MATERIALS AND METHODS

#### Oysters

This study was undertaken as part of a farming study conducted by NSW Department of Primary Industries (Nell & Perkins 2005). The triploids were produced by fertilization of eggs from diploids with sperm from tetraploids (Guo et al. 1996). Oysters were grown on an intertidal lease in Cromarty Bay, Port Stephens from May 2002 to October 2003 (Nell & Perkins 2005), where the salinity at harvest was 35‰. The market size for Pacific oysters in NSW is around 80 g (86 mm shell height). Because the triploids grew much faster than the diploids (Nell & Perkins 2005), diploids and triploids could not be matched for size. The average whole weight and shell height for the diploid and triploid oysters were 84 g (88 mm) and 101 g (91 mm), respectively.

Immediately after harvesting, oysters were depurated in flow-through tanks in UV sterilized seawater for 36 h. Oysters were packed in ice and transported by air from Port Stephens to Food Science Australia's (FSA) Werribee Center (Victoria) for taste testing. For ease of operation oysters were pooled for Condition Index (CI) determination. Four representative samples of six oysters of each type (diploid or triploid) were used to estimate the whole oyster weight, dry and wet meat weight and CI of oysters used in the taste test. The meats were dried at 90°C for 48 h then transferred to desiccators to cool before determining final dry weight. CI was calculated using the following formula (Crosby & Gale 1990):

$$CI = \frac{\text{dry meat weight (g)} \times 1000}{\text{cavity volume (g)}} \text{ where:} \\ \text{Cavity volume} = \text{whole weight (g)} - \text{shell weight (g)} \\ \text{(Lawrence \& Scott 1982).}$$

Because triploid oysters may suffer discoloration in summer (Hand & Nell 1999, Nell & Perkins 2005) the consumer taste tests were carried out in spring, before triploid Pacific oysters from Port Stephens suffer this problem.

#### Experimental Treatments

The experimental treatments were: diploid and triploid untreated controls, HP treated diploid and triploid oysters without salt

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and HP treated diploid and triploid oysters with salt. Prior to HP treatment, oysters were washed, clasped with a rubber band and placed in plastic bags filled with either fresh water or 3% saline solution (NaCl on a weight for volume basis in water). Oysters were HP processed in a 35 L HP processing unit (Quintus Press, Avure Technologies Inc, Kent, WA) for 3 min at 275 Mpa. This process shucked the oysters, but their integrity was maintained with the rubber band. After processing, oysters were stored on ice in a 4°C controlled temperature refrigerator for four days. The untreated (control) oysters were washed and stored as described above until the sensory evaluation session.

#### Consumer Sensory Evaluation

The consumer sample consisted of 61 regular consumers of oysters between the ages of 20 and 60 y from the Melbourne, Victoria, metropolitan area. The sample was divided into four 10-year age groups, each consisting of approximately equal numbers of male and female respondents. Prior to the test, respondents were briefed on the purpose of the trial and signed a participation consent form. Respondents were then instructed on the use of measurement scales and interstimulus palate cleaners. For the purpose of controlled product evaluation, each respondent was seated in an individual sensory booth. The untreated (control) oysters were shucked immediately prior to evaluation and the rubber band was removed from the HP treated oysters. All oysters were presented to respondents on a bed of ice (approx. 4°C) in plastic odorless disposable containers labeled with 3-digit codes. Respondents were instructed to eat the whole oyster. Test products were evaluated monadically (each respondent sees and evaluates only one product at a time in a sequential manner), and order of product presentation was balanced according to a row-column design. Hedonic (pleasure related) appraisal of odor, appearance, flavor, saltiness, texture and overall liking was rated on a 10-cm unstructured line scale anchored at 0 and 10 cm with “dislike extremely” and “like extremely,” respectively. Diagnostic ratings of saltiness (perceived salt taste intensity) were then measured using an unstructured line scale anchored at 0 and 10 cm with “not all salty” and “extremely salty,” respectively. Finally, respondents rated their “ideal” level of saltiness on an unstructured line scale anchored at 0 and 10 cm with “not all salty” and “extremely salty,” respec-

tively. All data was recorded and stored using a sensory data acquisition program (Compusense Inc., Guelph, Ont.).

#### Data Analysis

One-way analysis of variance (ANOVA; SPSS v 14; SPSS Australasia Pty Ltd., North Sydney, NSW) was used to compare individual products ( $n = 6$ ), process treatments ( $n = 3$ ) and oyster ploidy ( $n = 2$ ). Duncan's *post-hoc* test for means separation was used to determine the nature of discrimination between individual products and between treatments based on significant sensory and affective information. Mean CI values were also compared using one-way ANOVA. Data are expressed as means  $\pm$ SD throughout the text. Statistical significance (i.e., differences between products) was established at  $P < 0.05$ .

### RESULTS

#### Individual Products

Mean hedonic and diagnostic sensory attribute ratings for individual products are outlined in Table 1. There was large variation between individual consumer responses as reflected by the magnitude of the SD (Table 1). No differences were identified between individual products for consumer appreciation of odor, flavor, texture, aftertaste or overall liking. The triploid untreated oysters received a lower rating for appearance ( $F [5357] = 6.67; P < 0.01$ ) compared with the untreated and HP treated diploid oysters. Furthermore, the appearance of the triploid HP treated oysters was rated lower ( $F [5357] = 6.67; P < 0.01$ ) than that of the HP treated diploid oysters. Consumers preferred the saltiness [ $F (5357) = 5.24; P < 0.01$ ] of the diploid and triploid oysters HP treated without saline compared with the untreated products. Furthermore, consumers preferred the saltiness ( $F [5357] = 5.24; P < 0.01$ ) of the diploid and triploid oysters HP treated with or without saline compared with the untreated triploid oyster. The untreated triploid and diploid oysters were perceived to be saltier ( $F [5357] = 11.97; P < 0.01$ ) than the HP treated triploid and diploid oysters (processed with or without saline).

#### Processing Treatments

Mean hedonic and diagnostic sensory attributes ratings for processing treatments are outlined in Table 2. There was large varia-

TABLE 1.  
Hedonic and diagnostic sensory attribute ratings\* for individual oyster products.

Sensory Attributes	Individual Products					
	Triploid Untreated	Diploid Untreated	Triploid HP	Diploid HP	Triploid HP (3% Saline)	Diploid HP (3% Saline)
<i>Hedonic</i>						
Odor	6.24 $\pm$ 1.99 <sup>a</sup>	6.67 $\pm$ 2.06 <sup>a</sup>	6.36 $\pm$ 2.09 <sup>a</sup>	6.47 $\pm$ 2.28 <sup>a</sup>	6.41 $\pm$ 2.01 <sup>a</sup>	6.11 $\pm$ 2.10 <sup>a</sup>
Appearance	5.43 $\pm$ 2.17 <sup>a</sup>	6.53 $\pm$ 2.39 <sup>bc</sup>	6.23 $\pm$ 2.34 <sup>ab</sup>	7.22 $\pm$ 2.04 <sup>c</sup>	6.00 $\pm$ 2.33 <sup>ab</sup>	7.31 $\pm$ 1.74 <sup>c</sup>
Flavor	5.42 $\pm$ 2.42 <sup>a</sup>	5.68 $\pm$ 2.87 <sup>a</sup>	5.88 $\pm$ 2.63 <sup>a</sup>	6.25 $\pm$ 2.63 <sup>a</sup>	6.26 $\pm$ 2.34 <sup>a</sup>	6.18 $\pm$ 2.30 <sup>a</sup>
Saltiness	4.42 $\pm$ 2.51 <sup>a</sup>	4.97 $\pm$ 2.62 <sup>ab</sup>	5.93 $\pm$ 2.08 <sup>c</sup>	6.26 $\pm$ 2.09 <sup>c</sup>	5.67 $\pm$ 2.24 <sup>bc</sup>	5.69 $\pm$ 2.12 <sup>bc</sup>
Texture	6.22 $\pm$ 1.86 <sup>a</sup>	6.28 $\pm$ 2.62 <sup>a</sup>	6.01 $\pm$ 2.66 <sup>a</sup>	6.21 $\pm$ 2.51 <sup>a</sup>	6.29 $\pm$ 2.25 <sup>a</sup>	6.37 $\pm$ 2.13 <sup>a</sup>
Aftertaste	5.46 $\pm$ 2.43 <sup>a</sup>	5.56 $\pm$ 2.76 <sup>a</sup>	6.11 $\pm$ 2.32 <sup>a</sup>	6.00 $\pm$ 2.67 <sup>a</sup>	5.92 $\pm$ 2.45 <sup>a</sup>	5.53 $\pm$ 2.48 <sup>a</sup>
Overall liking	5.16 $\pm$ 2.39 <sup>a</sup>	5.76 $\pm$ 2.65 <sup>a</sup>	5.96 $\pm$ 2.51 <sup>a</sup>	6.24 $\pm$ 2.55 <sup>a</sup>	5.88 $\pm$ 2.56 <sup>a</sup>	5.95 $\pm$ 2.44 <sup>a</sup>
<i>Diagnostic</i>						
Saltiness intensity	6.62 $\pm$ 2.37 <sup>a</sup>	6.18 $\pm$ 2.34 <sup>a</sup>	3.99 $\pm$ 2.30 <sup>b</sup>	4.57 $\pm$ 2.42 <sup>b</sup>	4.58 $\pm$ 2.35 <sup>b</sup>	4.65 $\pm$ 2.31 <sup>b</sup>
Ideal saltiness intensity	4.69 $\pm$ 2.19 <sup>a</sup>	4.83 $\pm$ 2.13 <sup>a</sup>	4.47 $\pm$ 2.14 <sup>a</sup>	4.54 $\pm$ 2.14 <sup>a</sup>	4.75 $\pm$ 2.08 <sup>a</sup>	4.55 $\pm$ 2.11 <sup>a</sup>

\* Data are expressed as means  $\pm$  SD. Within rows, means with different letters differ significantly ( $p < 0.05$ ).



TABLE 2.

Hedonic and diagnostic sensory attribute ratings\* for oyster processing treatments.

Hedonic Scores	Processing Treatments		
	Untreated	HP	HP (3% Salt)
<i>Hedonic</i>			
Odor	6.46 ± 2.03 <sup>a</sup>	6.41 ± 2.18 <sup>a</sup>	6.26 ± 2.05 <sup>a</sup>
Appearance	5.98 ± 2.34 <sup>a</sup>	6.73 ± 2.24 <sup>b</sup>	6.65 ± 2.15 <sup>b</sup>
Saltiness	4.70 ± 2.57 <sup>a</sup>	6.10 ± 2.08 <sup>b</sup>	5.68 ± 2.17 <sup>b</sup>
Flavor	5.55 ± 2.64 <sup>a</sup>	6.07 ± 2.63 <sup>a</sup>	6.22 ± 2.31 <sup>a</sup>
Texture	6.25 ± 2.26 <sup>a</sup>	6.11 ± 2.58 <sup>a</sup>	6.33 ± 2.18 <sup>a</sup>
Aftertaste	5.51 ± 2.59 <sup>a</sup>	6.06 ± 2.49 <sup>a</sup>	5.73 ± 2.47 <sup>a</sup>
Overall liking	5.46 ± 2.53 <sup>a</sup>	6.10 ± 2.52 <sup>a</sup>	5.91 ± 2.49 <sup>a</sup>
<i>Diagnostic</i>			
Saltiness intensity	6.40 ± 2.35 <sup>a</sup>	4.28 ± 2.37 <sup>b</sup>	4.61 ± 2.32 <sup>b</sup>
Ideal saltiness intensity	4.76 ± 2.15 <sup>a</sup>	4.51 ± 2.17 <sup>a</sup>	4.65 ± 2.09 <sup>a</sup>

\* Data are expressed as means ± SD. Within rows, means with different letters differ significantly ( $p < 0.05$ ).

tion between individual consumer responses as reflected by the magnitude of the SD (Table 2). Consumers did not convey differences between processing treatments for appreciation of oyster odor, flavor, texture, aftertaste or overall liking. Consumers rated the appearance of the untreated oysters lower ( $F [2360] = 4.10$ ;  $P < 0.05$ ) than that of the HP treated oysters. Consumers also preferred the saltiness ( $F [2360] = 11.93$ ;  $P < 0.01$ ) of the HP treated oysters compared with the untreated oysters. In terms of actual perceived saltiness intensity, the untreated oysters received higher ratings ( $F [2360] = 28.51$ ;  $P < 0.01$ ) compared with the HP treated oysters.

#### Oysters Type

Mean hedonic and diagnostic sensory attributes ratings for each type of oyster are outlined in Table 3. There was large variation between individual consumer responses as reflected by the magnitude of the SD (Table 3). Consumers did not discern differences between diploid and triploid oysters for liking of odor, flavor, texture, aftertaste, overall liking or saltiness. Furthermore, there was no difference between oysters for consumer appraisal of per-

TABLE 3.

Hedonic and diagnostic sensory attribute ratings\* for oyster type.

Sensory Attributes	Oyster Type		Significance
	Diploid	Triploid	
<i>Hedonic</i>			
Odor	6.42 ± 2.15	6.33 ± 2.02	ns
Appearance	7.02 ± 2.09	5.88 ± 2.30	$p < 0.01$
Saltiness	5.64 ± 2.33	5.34 ± 2.37	ns
Flavor	6.03 ± 2.61	5.85 ± 2.47	ns
Texture	6.29 ± 2.42	6.17 ± 2.27	ns
Aftertaste	5.70 ± 2.63	5.83 ± 2.41	ns
Overall liking	5.98 ± 2.54	5.66 ± 2.50	ns
<i>Diagnostic</i>			
Saltiness intensity	5.14 ± 2.46	5.07 ± 2.59	ns
Ideal saltiness intensity	4.64 ± 2.15	4.64 ± 2.13	ns

\* Data are expressed as means ± SD.

ceived saltiness intensity. However, consumers did prefer the appearance of the diploid oysters ( $F [1361] = 24.08$ ;  $P < 0.01$ ). From a physiological perspective, there were differences in whole weight, dry meat weight and CI between the diploid and triploid oysters (Table 4).

#### DISCUSSION

This study showed similar consumer acceptance measures for triploid and diploid oysters. This finding is in agreement with previous hedonic comparisons of diploid and triploid Pacific oysters by consumer panels (Allen & Downing 1991, Maguire et al. 1994, Chao et al. 2001). Similarly, Korac et al. (1996) found relatively similar consumer acceptance ratings for triploid and diploid Sydney rock oysters. Notwithstanding this, in the present study consumers did prefer the appearance of the diploid oysters despite the processing treatment. It is likely that this was caused by the lower CI of the triploid (CI of 84) compared with the diploid (CI of 133) oysters. Ideally, this study should have been done on oysters of similar meat condition, but this is difficult because the CI of diploids (50–150) fluctuates greatly, whereas that of triploids is more stable (70–110) (Nell & Perkins 2005). The main advantage of triploidy in NSW is faster growth and better meat condition over late spring and early autumn when diploids spawn, outside of the possible midspring to autumn period of gonad discoloration (Nell & Perkins 2005).

HP did not have a negative impact on the eating quality of triploid and diploid oysters. Conversely, HP actually improved the appearance of the triploid and diploid oysters, which is in agreement with previous studies (López-Caballero et al. 2000). HP also seemed to reduce the intensity of perceived saltiness in oysters. This effect was appealing to consumers and measurement of their ideal saltiness intensity was similar to that perceived in the HP treated oysters (Tables 1, 2). HP may have reduced the salt content of the oyster samples because processing took place in an aqueous medium and osmotic movement of salt from the oyster to the processing medium may have occurred. However, the salt ion concentration of the oyster and processing medium was not measured before and after processing. Whereas observations based on perceived saltiness were in contrast with a previous report by Hoover et al. (1989), differences in the levels of oyster saltiness observed in the present study did not impact on measures of overall liking.

HP showed considerable promise as a means of improving the sensory quality (appearance and saltiness) of oysters. HP inactivates microorganisms while maintaining sensory and nutritional qualities (Murchie et al. 2005). Specifically, HP reduces the levels of *Vibrio vulnificus* in oysters but little is known of its effect on human enteric viruses (Murchie et al. 2005). As well as investigating efficacy in reducing the levels of human enteric viruses

TABLE 4.

Physiological measurements\* of oyster type.

Physiological Measurements	Oyster Type		Significance
	Diploid	Triploid	
Whole oyster weight (g)	84.2 (7.4)	100.9 (3.0)	$p < 0.01$
Dry meat weight (g)	3.8 (0.5)	2.9 (0.6)	$p < 0.05$
Condition Index	132.8 (15.9)	83.9 (13.3)	$p < 0.001$

\* Data are expressed as means ± SD.

(Murchie et al. 2005), future studies should also investigate the potential of HP to extend the shelf life of refrigerated oysters (He et al. 2002). This would have important implications from a marketing perspective in terms of product positioning, market access and distribution. Development of oysters from a marketing perspective is imperative (Liu et al. 2006) because demand for this product is strong (Ruello & Associates 2002, 2005).

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## ERRATUM

### TAQMAN<sup>®</sup> MGB REAL-TIME PCR APPROACH TO QUANTIFICATION OF *PERKINSUS MARINUS* AND *PERKINSUS* SPP. IN OYSTERS

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TABLE 1.

Primer and probe sequences for PERK and PMAR TaqMan<sup>®</sup> MGB assays. Probes include a 5' 6-carboxyfluorescein (FAM) reporter and 3' MGB.

<i>Perkinsus</i> spp. (PERK) Assay:
Forward: 5'-TCCGTGAACCAGTAGAAATCTCAAC-3'
Reverse: 5'-GGAAGAAGAGCGACACTGATATGTA-3'
Probe: 5'-CCCTTTGTGCAGTATGC-MGB-3'
<i>Perkinsus marinus</i> (PMAR) Assay:
Forward: 5'-TTGTTAACGCAACTCAATGCTTTGT-3'
Reverse: 5'-AAGCGCACATAACGAACCACC-3'
Probe: 5'-GCTTGAACCTAACTCT-MGB-3'

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**COVER PHOTO:** Shellfish provide more than a food crop in many countries. Shell art is a staple of the global tourist industry—this photo taken at La Ballena Gris in Comondú, Baja California Sur, Mexico. Shells from locally collected and consumed species as well as imported shells were used in these trinkets which were assembled on site. (Photo: S. E. Shumway)

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